EXPERT REPORT OF PROFESSOR DENNIS R. WINGE

ON THE ISSUE OF INVALIDITY

MAY 26, 2017

I. INTRODUCTION

I, Dennis R. Winge, have been retained as an expert witness on behalf of Defendants Lallemand Specialties, Inc. and Mascoma LLC (to whom I will refer collectively as Lallemand). If called to testify at hearing or trial, I expect to give testimony concerning my opinions described in this report.

1) I submit this statement of – and the factual bases for – my opinions. The opinions and facts contained in this report are based in part on information made available to me in this case on or before the date of this report. I expressly reserve the right to supplement or modify this report if and when I acquire additional relevant information before trial.

2) I have been asked by counsel for Lallemand to provide my opinions regarding whether each of the asserted claims (i.e., claims 1, 5, and 11) of U.S. Patent No. 8,795,998 (the ‘998 patent) is disclosed in one or more prior art references, or is rendered obvious by combinations of one or more prior art references and/or the ordinary level of knowledge and skill of a person having ordinary skill in the art (POSITA). I have also been asked for my opinion regarding whether a POSITA would be able to ascertain the scope of the asserted claims of the ‘998 patent with reasonable certainty, and whether a POSITA could find adequate support for the alleged scope of the asserted claims in the written description contained within the ‘998 patent specification. This report contains my opinions regarding these issues and the bases for them.

A. Background and Qualifications

3) My PhD training at Duke University was in biochemistry, and since starting my independent academic research laboratory at the University of Utah in 1979 I have

broadened my expertise to include molecular biology and genetics.

4) I have been continuously funded by the National Institutes of Health from 1980 to the present.

5) I have published over 210 manuscripts in peer-reviewed journals. I have over 72 publications with over 70 citations and have an H-index of 72. I have published multiple papers with alcohol dehydrogenase as a topic.

6) I am a member of three editorial boards including the Journal of Biological Chemistry, which is one of the most prominent biochemistry journals. In the past couple of years I have reviewed for most top tier journals including Cell, Nature, Cell Metabolism and the Proceedings of the National Academy of Sciences. I have reviewed manuscripts related to cellular redox control, and this topic is of interest to me.

7) My research has been focused in two broad areas, namely metallobiochemistry and mitochondrial biology. We have worked predominantly with yeast as an experimental system using *Saccharomyces cerevisiae*. The bulk of my research publications have been with this yeast.

8) From 1979 to the mid-1990s my research focused on how yeast maintain nutrient homeostasis with a focus on critical metal ions such as iron, zinc, copper and manganese. From the mid-1990s we switched our focus to studying mitochondrial biology. Since oxidative phosphorylation is a dominant type of metabolism in most aerobic life forms, we were focused on how yeast assemble the various respiratory complexes involved in oxidative phosphorylation. In our research, we frequently used glycerol as a carbon source to assess the capacity of yeast to respire. As such, I am very familiar with metabolic pathways involving glycerol. Likewise, our studies also

utilized acetate as a carbon source to study mutations in succinate dehydrogenase assembly mutants. Thus, I am very familiar with metabolic pathways involving acetate.

9) Since we utilized yeast as our experimental system, we routinely cultured the organism under fermentation conditions in order to study genes essential to oxidative phosphorylation. Thus, I am very familiar with both fermentative and respiratory metabolism in yeast and understand the critical importance in maintaining redox homeostasis as well as nutrient homeostasis in cell viability. My laboratory uses a combination of biochemistry, yeast biology and genetics to address questions related to mitochondrial function.

10) A copy of my complete *curriculum vitae* is attached to this report as Exhibit A.

B. Publications Authored in the Previous 10 Years

11) A list of my publications, including those I authored in the previous 10 years, is included with my *curriculum vitae*.

C. Testimony within Previous Four Years

12) I served as an expert witness in a patent law case involving Butamax v. Gevo in which patents were filed using *Saccharomyces cerevisiae* for biofuel production. My deposition in this case occurred on November 21, 2012.

D. Fees

13) I am an independent consultant, and I receive compensation of $350/hour, plus reimbursement for any expenses incurred. My compensation does not depend on the opinions I express in this report, my testimony, or the outcome of this litigation.

E. Preparation and Basis for Opinions

14) Besides conferring with counsel for Lallemand regarding the legal standards I am to apply in forming my opinions, I have performed a number of tasks in preparation for this expert report. I have been given access to all of the documents produced in this case. I have studied the ‘998 patent, including the asserted claims. I have examined the prosecution history of the ‘998 patent. I have reviewed and studied the prior art produced in this litigation. I have also reviewed the plaintiff's infringement contentions (including supplements) and Lallemand's invalidity contentions. I have also applied my own knowledge of microbial metabolic pathways that I have gained over the course of my research career as a biochemist.

15) Accordingly, I have studied the ‘998 patent and the prior art references, and I have been instructed on the relevant law. These resources have placed me in a position in which I can opine about the validity of the ‘998 patent in light of the prior art. Using the information I have gained from the above activities, I compared the prior art produced in this case to the asserted claims and formed opinions regarding the validity of the asserted claims in light of the prior art. Those opinions, and the bases for them, are set forth below.

16) In presenting the matters set forth in this report at trial, I may use some or all of the documents identified in this section and in attached exhibits, or excerpts and/or enlargements of them. Citations to various sections of documents in this report are not exhaustive, and I reserve the right to cite additional portions of these documents. I may also use deposition and trial testimony and the results of any testing or inspection of the accused TransFerm Yield+ accused products. I may also use visual aids and

demonstrative exhibits to help me explain my opinions, the bases thereof and testimony.

17) I expressly reserve the right to review additional materials, to supplement my report, and revise the opinions I will present at the trial based on any additional reports and testimony of the plaintiffs' experts, any additional discovery that might occur, further research on the subjects addressed in this report, and testimony and exhibits I observe at trial.

II. SUMMARY OF OPINIONS

18) The analyses concerning my opinions set forth in Sections III through VI, below, are necessarily detailed and voluminous. Therefore, to avoid confusion, here I provide the following summaries of my opinions, the bases for which are fully set forth in the Sections below.

A. Summary of Opinions Regarding Anticipation

19) It is my opinion that asserted claims 1, 5, and 11 are anticipated by WO2009/111672 ("Sun") under 35 U.S.C. §102(e).

B. Summary of Opinions Regarding Obviousness

20) It is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Nevoigt '270 in view of Wahlbom and/or Mueller and/or Sonderegger II and further in view of Taherzadeh. Alternatively, it is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Nevoigt '270 in view of Wahlbom and/or Mueller and/or Sonderegger II.

21) It is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Zhang '08 in view of Wahlbom and/or Mueller and/or Sonderegger II and further in view of Taherzadeh. Alternatively, it is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Zhang '08 in view of Wahlbom and/or Mueller and/or Sonderegger II.

22) It is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Nissen in view of Wahlbom and/or Mueller and/or Sonderegger II and further in view of Taherzadeh. Alternatively, it is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Nissen in view of Wahlbom and/or Mueller and/or Sonderegger II.

23) It is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Guo in view of Wahlbom and/or Mueller and/or Sonderegger II and further in view of Taherzadeh. Alternatively, it is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Guo in view of Wahlbom and/or Mueller and/or Sonderegger II.

24) It is my opinion that asserted claims 1, 5, and 11 are rendered obvious under 35 U.S.C. §103 over Valadi '98 in view of Sonderegger if the claims are construed such that the claimed transgenic yeast cells do not require acetate in order to grow.

C. Summary of Opinions Regarding Indefiniteness

25) It is my understanding from my review of the materials provided to me in this case that the plaintiffs' infringement contentions adopt a claim interpretation that ignores the function of "one or more recombinant heterologous, nucleic acid sequences

encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10)" (which I will refer to as the "AADH requirement"). From my review of the '998 patent specification and prosecution history, it is clear to me that the only purpose for the AADH requirement disclosed by the inventors is to "allow[] the re-oxidation of NADH when acetyl-coenzyme A is generated from acetate present in the growth medium." If the yeast cells of claim 1 do not require the addition of acetate in the growth medium in order to grow, it is my opinion that the claimed invention is not described in the '998 patent specification such that a POSITA would understand that the inventors had actually invented what is claimed.

26) The Asserted Claims require, among other things, "wherein said cells lack enzymatic activity needed for the NADH-dependent glycerol synthesis, or said cells have a reduced enzymatic activity with respect to the NADH-dependent glycerol synthesis compared to a corresponding wild-type yeast cell." It is my understanding that the inventors measured such decreased (or null) enzymatic activity in the cells of their invention solely by using an assay described in a paper by Blomberg and Adler. See Col. 20, lns. 35-41. It is also my understanding that Lallemand performed the Blomberg and Adler assay on the accused yeast cells and demonstrated an increased, rather than decreased, enzymatic activity. It is also my understanding that these results were shared with DSM, but that DSM dismissed the data as irrelevant to the infringement analysis. If DSM's position is correct, a POSITA attempting to determine whether or not a particular transgenic yeast strain infringes the claim would be unable to do so because any results of the very assay disclosed in the '998 patent would be irrelevant to such determination, because results that show either decreased or no change in

enzymatic activity would nevertheless meet this claim limitation so long as the cells have decreased glycerol production for any reason. It is therefore my opinion that asserted claims 1, 5, and 11 are indefinite for failing to satisfy the written description requirement of 35 U.S.C. §112, ¶1, and/or for failing to particularly point out and distinctly claim the invention as required by 35 U.S.C. §112, ¶2, and are therefore invalid.

III. OVERVIEW OF U.S. PATENT NO. 8,795,998

27) The '998 patent claims priority to a European patent application dated July 24, 2009 and is titled "Fermentative Glycerol-Free Ethanol Production." By way of further introduction, the Abstract states that:

> The present invention relates to a yeast cell, in particular a recombinant yeast cell, the cell lacking enzymatic activity needed for the NADH-dependent glycerol synthesis or the cell having a reduced enzymatic activity with respect to the NADH-dependent glycerol synthesis compared to its corresponding wild-type yeast cell, the cell comprising on or more heterologous nucleic acid sequences encoding an NAD^+-dependent acetylating acetaldehyde dehydrogenase (EC 1.2.1.10) activity. The invention further relates to the use of a cell according to the invention in the preparation of ethanol.

28) As implied by the title, the '998 patent is directed to the production of ethanol via fermentation by recombinant yeast cells. See Col. 1, lns. 29-36. The inventors defined "recombinant (cell)" as:

> a strain (cell) containing nucleic acid which is the result of one or more genetic modifications using recombinant DNA technique(s) and/or another mutagenic technique(s). In particular a recombinant cell may comprise nucleic acid not present in a corresponding wild-type cell, which nucleic acid has been introduced into that strain (cell) using recombinant DNA techniques (a transgenic cell), or which nucleic acid not present in said wild-type is the result of one or more mutations--for example using recombinant DNA techniques or another mutagenesis technique such as

> UV-irradiation--in a nucleic acid sequence present in said wild-type (such as a gene encoding a wild-type polypeptide) or wherein the nucleic acid sequence of a gene has been modified to target the polypeptide product (encoding it) towards another cellular compartment. Further, the term "recombinant (cell)" in particular relates to a strain (cell) from which DNA sequences have been removed using recombinant DNA techniques.

Col. 4, In. 64 through Col. 5, In. 14.

29) As background, the inventors noted a then-current research effort to expand the range of fermentation substrates to include "lignocellulosic biomass from non-food feedstocks (e.g., energy crops and agricultural residues, forestry residues or industrial/consumer waste materials that are rich in cellulose, hemicellulose, and/or pectin) and to increase productivity, robustness and product yield." Col. 1, lns. 36-43. The authors also stated that "it is an object of the invention to provide a novel recombinant cell, which is suitable for the anaerobic, fermentative production of ethanol from a carbohydrate, in particular a carbohydrate obtained from lignocellulosic biomass,…." Col. 2, lns. 34-37.

30) The inventors also identified a problem that had to be overcome in order to achieve their objective:

> Lignocellulosic biomass is abundant, however is in general not readily fermented by wild-type ethanol producing micro-organisms, such as *S. cerevisiae*. The biomass has to be hydrolysed. The resultant hydrolysate is often a mixture of various monosaccharides and oligosaccharides, which may not all be suitable substrates for the wild-type micro-organism. Further, the hydrolysates typically comprise acetic acid, formed as a by-product in particular when hydrolysing pectin or hemicellulose, and--dependent on the type of hydrolysis--one or more other by-products or residual reagents that may adversely affect the fermentation. In particular, acetic acid has been reported to negatively affect the kinetics and/or stoichiometry of sugar fermentation by wild-type and genetically modified *S. cerevisiae* strains and its toxicity is strongly augmented at low culture pH

Col. 1, lns. 44-58. I may refer to this below as the “acetate problem.” In addition, for purposes of this report, unless otherwise noted I will attempt to use the inventors’ definitions so that usage is consistent. Hence, when I refer to “acetate” in the context of the ‘998 patent, I mean to refer to the corresponding carboxylic acid (i.e., acetic acid), and *vice versa*, depending on the context. See Col. 4, lns. 36-38.

31) Another problem (i.e., a “major challenge”) identified by the inventors regarding industrial ethanol fermentation is the formation of glycerol as an unwanted byproduct, because it both reduces overall conversion of sugar to ethanol, and imposes potential costs for waste-water treatment. Col. 2, lns. 3-33. Glycerol formation under anaerobic conditions in yeast such as *S. cerevisiae* apparently exists in part to rebalance the ratio of NAD^+ to NADH. *Id.* I may refer to this below as the “glycerol problem.”

32) The inventors purported to have solved simultaneously both the acetate problem and the glycerol problem “by providing a specific recombinant cell wherein a specific other enzymatic activity has been incorporated, which allows re-oxidation of NADH formed in the fermentation of a carbohydrate, also in the absence of enzymatic activity needed for the NADH-dependent glycerol synthesis.” Col. 2, lns. 49-55. In this way, the inventors asserted that:

> The present invention allows complete elimination of glycerol production, or at least a significant reduction thereof, by providing a recombinant yeast cell, in particular *S. cerevisiae*, such that it can reoxidise NADH by the reduction of acetic acid to ethanol via NADH-dependent reactions.

Col. 3, lns. 56-60.

33) The inventors purported to solve the glycerol problem by eliminating or reducing "enzymatic activity needed for the NADH-dependent glycerol synthesis." Col. 9, lns. 24-28. According to the inventors:

> A reduced enzymatic activity can be achieved by modifying one or more genes encoding a NAD-dependent glycerol 3-phosphate dehydrogenase activity (GPD) or one or more genes encoding a glycerol phosphate phosphatase activity (GPP), such that the enzyme is expressed considerably less than in the wild-type or such that the gene encoded a polypeptide with reduced activity. Such modifications can be carried out using commonly known biotechnological techniques, and may in particular include one or more knock-out mutations or site-directed mutagenesis of promoter regions or coding regions of the structural genes encoding GPD and/or GPP. Alternatively, yeast strains that are defective in glycerol production may be obtained by random mutagenesis followed by selection of strains with reduced or absent activity of GPD and/or GPP. *S. cerevisiae* GDP 1, GDP2, GPP1 and GPP2 genes are shown in SEQ ID NO: 24-27.

Col. 9, lns. 35-50. Thus, according to the inventors, the glycerol problem can be solved in accordance with the invention by modifying one or more genes encoding GPD or GPP, such that the enzyme is (1) expressed considerably less than in the wild type or (2) such that the gene encodes a polypeptide with reduced activity (this can only occur if one uses a mutation within the coding region to compromise catalytic activity).

34) The inventors purport to have solved the acetate problem by incorporating a heterologous gene encoding a "specific other enzymatic activity" identified as "an NAD^+-dependent acetylating acetaldehyde dehydrogenase (EC 1.2.1.10) activity." Col. 2, lns. 56-60. I note that this specific other enzymatic activity is incorporated into claim 1 essentially *verbatim*. Col. 67, lns. 14-15. The specific purpose for the introduction of this gene is to allow "the re-oxidation of NADH when acetyl-Coenzyme A is generated from acetate present in the growth medium." Acetate is converted to acetyl-CoA by acetyl

CoA synthetase and acetyl CoA is converted to acetaldehyde by acetaldehyde dehydrogenase (AADH) and finally alcohol dehydrogenase (ADH) generates ethanol and the regeneration of NAD^+. Col. 10, lns. 13-16. I see no other purpose for the incorporation of AADH into the recombinant yeast cells of the invention identified anywhere in the '998 patent.

35) The '998 patent includes two exemplary experiments. In the first example, the inventors constructed transgenic *S. cerevisiae* strains in which *GPD1* and *GPD2* were inactivated (RWB0094, IMZ008, IMZ127) and a yeast strain in which *GPD1* and *GPD2* were inactivated and an *mhpF* gene, encoding a bifunctional *E. coli* acetylating acetaldehyde dehydrogenase, was introduced (IMZ132). Col. 15, ln. 55 – Col. 19, ln. 10. According to the inventors, one of the *gpd1Δ/gpd2Δ* strains (it is unclear whether it was RWB0094, IMZ008, or IMZ127) failed to grow on glucose, confirming that glycerol production is essential for fermentation growth of *S. cerevisiae*. Col. 20, lns. 49-67; Fig. 2D. Additionally, the strain IMZ132 (containing the mhpF AADH gene) failed to grow on glucose in the absence of acetic acid. Col. 20, lns. 24-28; Fig. 2C. However, when the medium was supplemented with 2.0 g/L acetic acid, exponential growth was observed at a specific growth rate of 0.14/hr. Col. 20, lns. 28-30; Fig. 2C. As expected, glycerol formation was negligible and ethanol yield increased in this case. Col. 20, lns. 30-48; Fig. 2C. The inventors concluded that:

> The present study provides proof of principle that, stoichiometrically, the role of glycerol as a redox sink for anaerobic growth of *S. cerevisiae* can be fully replaced by a linear pathway for NADH-dependent reduction of acetate to ethanol. This offers interesting perspectives for large-scale ethanol production from feedstocks that contain acetic acid, such as lignocellulosic hydrolysates.

> In addition to reducing the organic carbon content of spent media and increasing the ethanol yield, the reduction of acetic acid to ethanol may at least partially alleviate acetate inhibition of yeast growth and metabolism, which is especially problematic at low pH and during the consumption of pentose sugars by engineered yeast strains

Col. 21, ln. 50 – Col. 22, ln. 2.

36) In the second example, the inventors constructed a *gpd1Δgpd2Δ S. cerevisiae* strain in which the dmpF gene encoding an AADH enzyme from a *Pseudomonas* strain was inserted. Growth on glucose in the presence of 2.0 g/L acetic acid was comparable to strain IMZ132 (containing the *E. coli* mhpF AADH gene). Col. 22, lns. 6-49. The inventors again concluded that:

> The insertion of a synthetic codon optimized copy of the gene dmpF from *Pseudomonas* sp. CF600, provides another example that it is stoichiometrically possible to substitute glycerol formation as a redox sink in anaerobic growth of S. cerevisiae by a linear metabolic pathway for NADH-dependent reduction of acetate to ethanol. Also this example shows that the insertion of (acetylating) acetaldehyde dehydrogenase in a gpd1Δ.gpd2Δ. *S. cerevisiae* strain resulted in higher ethanol yields on glucose, no formation of by-product glycerol, and the consumption of fermentation inhibitor compound acetate.

Col. 22, lns. 50-60.

37) I note that the inventors did not include any experiments in which only one of *GPD1* or *GPD2* was deleted, or what effect that would have on their acetate consuming strain. As I mentioned above, the literature at the time suggested that deletion of *GPD1* has a negligible effect on glycerol formation under fermentative, non-osmotically stressed conditions, while deletion of *GPD2* leads to overexpression of Gpd1, at least under certain conditions. See Valadi '98, Ansell, and Nissen. It would be interesting to determine what effect the '998 patent's AADH pathway would have on a strain that has a functioning (albeit disrupted) glycerol pathway. Theoretically, it is possible that the

AADH pathway would shunt carbon to acetyl-CoA at the expense of the more energetically costly glycerol pathway, leading to reduced glycerol formation without reducing GDH enzymatic activity (as determined by the Blomberg and Adler assay set forth by the '998 patent inventors). Alternatively, continued glycerol production could tend to balance cytosolic [NAD^+/NADH] equilibrium in competition with the AADH pathway, possibly affecting the NAD^+-dependent acetylating acetaldehyde dehydrogenase reaction by pushing it in the direction toward formation of acetyl-CoA (and subsequently acetate), rather than acetaldehyde:

$$\text{Acetyl-CoA} + \text{NADH} + H^+ \leftrightarrow \text{acetaldehyde} + NAD^+ + \text{CoA}$$

In this case, the presence of acetate under these conditions could prove to be toxic to the inventors' strain, as less would be consumed.

38) As I mentioned above, as part of my work on this case I have reviewed the prosecution history of the '998 patent from the perspective of a POSITA. From this perspective I note that the claims were substantially amended during the course of prosecution. I review the more salient points relating to these amendments below.

39) In response to an obviousness rejection based on Sonderegger in view of Valadi, the inventors again stated that the "inventive nature of the ability of the claimed cells to utilize acetate" was what distinguished their invention from the prior art. May 2, 2013 Amendment Under 37 C.F.R. § 1.111, p. 7. To reiterate this salient point, the inventors asserted that their "invention provides a yeast cell that actually grows preferentially in the presence of acetate," and that their invention "takes advantage of the presence of acetate." *Id.*, pp. 7-8. The inventors further observed that even the examiner recognized

that “it is important that the present invention provides yeast that can consume acetate and turn it into ethanol.” Id., p. 8.

40) In response to the inventors’ arguments regarding the criticality of acetate to the invention, the examiner issued a final office action in which he allowed then-pending claims 7 and 12-15 on the basis that only those claims “include or imply th[e] distinguishing limitation” of acetate dependency. August 1, 2013 Final Office Action, p. 8. The examiner rejected the other pending claims on the basis that those claims did not “encompass all the structural and/or functional attributes recited in the specification.” Id.

41) In response to the Final Office Action, the inventors inserted the structural limitations of claim 7 into claim 1, thereby inserting what the examiner considered the structural “acetate requirement” limitation into that claim. October 30, 2013 Amendment Under 37 C.F.R. § 1.111, pp. 1-2. As a result, the Patent Office allowed the claims. November 25, 2013 Notice of Allowance. Claim 1, as amended, is currently-asserted claim 1 in this litigation.

IV. OPINIONS REGARDING ANTICIPATION

A. Legal Standards

42) My understanding of the law regarding anticipation has been informed by counsel for Lallemand. I am informed that a claim is invalid as anticipated if each and every limitation as set forth in the claim is described, expressly or inherently, in a single prior art reference. I am informed that a reference is considered prior art if it comprises information that was already publicly known or used by others anywhere in the world, or

was described anywhere in the world in a printed publication, prior to the alleged invention set forth in the claim.

43) I am also informed that a reference is considered prior art if it was patented or described in a printed publication anywhere in the world, or was in public use or on sale in the United States and its territories more than one year prior to the filing date of the subject patent.

44) I am also informed that a reference is considered prior art if it was not abandoned suppressed, or concealed by another inventor before the invention date.

45) I am informed that a prior art reference may describe limitations in a subject claim explicitly or "inherently." A limitation is inherent in a prior art reference if a POSITA would have understood and appreciated that the prior art reference necessarily included the limitation in question, even if it was not expressly disclosed.

46) Lastly, I am also informed that the legal standard for proving that a claim is anticipated is the "clear and convincing" standard of proof. I am informed that this standard is met when the jury possesses a firm belief or conviction that a fact is true.

B. Analysis

a. The Asserted Claims of U.S. Patent No. 8,795,998 Are Anticipated by Sun

47) The '998 patent claims priority to a European patent application filed on July 24, 2009. Therefore, I am informed by counsel for Lallemand that any patent or printed publication dated earlier than July 24, 2009 (the "critical date") is prior art to the '998 patent.

48) Sun has an international filing date of March 5, 2009, and was published on September 11, 2009. I am informed by counsel that this makes Sun prior art under 35 U.S.C. §102(e). I have compared the disclosure in Sun to each of the asserted claims of the ‘998 patent, and it is my opinion that Sun anticipates claims 1, 5, and 11. I reproduce those claims here, labeling each limitation individually for purposes of comparison with the prior art. In my analysis, I will use the labels to refer to the individual limitations:

Claim 1:

[a] Transgenic yeast cells comprising one or more recombinant heterologous, nucleic acid sequences encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10),

[b] wherein said cells lack enzymatic activity needed for the NADH-dependent glycerol synthesis, or said cells have a reduced enzymatic activity with respect to the NADH-dependent glycerol synthesis compared to a corresponding wild-type yeast cell, and

[c] wherein said cells are free of NAD-dependent glycerol 3-phosphate dehydrogenase activity or have reduced NAD-dependent glycerol 3-phosphate dehydrogenase activity compared to corresponding wild-type cells, and/or

[d] wherein the cells are either free of glycerol phosphate phosphatase activity or have reduced glycerol phosphate phosphatase activity compared to corresponding wild-type cells, and

[e] which comprise a genomic mutation in at least one gene selected from the group consisting of GPD1, GPD2, GPP1 and GPP2, and

[f] wherein said cells further comprise one or more nucleic acid sequences encoding an acetyl-Coenzyme A synthetase activity (EC 6.2.1.1) and

[g] one or more nucleic acid sequences encoding NAD^+-dependent alcohol dehydrogenase activity (EC 1.1.1.1).

Claim 5:

The cells of claim 1 which are **[h]** *Saccharomycetaceae*, *Kluyveromyces*, *Pichia*, *Zygosaccharomyces*, or *Brettanomyces*.

Claim 11:

The cells of claim 1, wherein **[i]** at least one said mutation is a complete deletion of said gene in comparison to the corresponding wild-type yeast gene.

49) Sun discloses limitation [a]. See p. 30, ln. 27 – p. 31, ln. 2 (listing non-naturally occurring, e.g., transgenic, yeast strains compatible with Sun’s invention); pp. 2, lns. 23-24; p. 60, lns. 6-10; p. 67, lns. 15-25.

50) Sun discloses disrupting a cytosolic NADH-linked glycerol-3-phosphate dehydrogenase. See p. 59, ln. 24 – p. 60, ln. 5; p. 61, ln. 23 – p. 62, ln. 2; p. 65, lns. 23-27; p. 68, lns. 5-30. To the extent that DSM's position that such gene disruption and any correlated reduced glycerol formation (regardless of the actual cause of such reduced glycerol formation) demonstrates reduced enzymatic activity, Sun discloses limitation [b].

51) Sun discloses limitation [c]. See p. 59, ln. 24 – p. 60, ln. 5; p. 61, ln. 23 – p. 62, ln. 2; p. 65, lns. 23-27; p. 68, lns. 5-30.

52) Sun does not explicitly disclose limitation [d], but that limitation is expressed in the alternative to limitation [c], which is disclosed. See p. 59, ln. 24 – p. 60, ln. 5; p. 61, ln. 23 – p. 62, ln. 2; p. 65, lns. 23-27; p. 68, lns. 5-30.

53) Sun discloses limitation [e]. See p. 59, ln. 24 – p. 60, ln. 5; p. 61, ln. 23 – p. 62, ln. 2; p. 65, lns. 23-27; p. 68, lns. 5-30.

54) Sun discloses limitations [f] and [g]. See p. 61, lns. 1-22; p. 62, lns. 13-14, and p. 114, lines 2-9.

55) Sun discloses limitation [h]. See p. 30, ln. 27 – p. 31, ln. 2.

56) Sun discloses limitation [i]. See p. 59, ln. 24 – p. 60, ln. 5; p. 61, ln. 23 – p. 62, ln. 22; p. 65, lns. 23-27; p. 68, lns. 5-30.

57) Accordingly, because Sun discloses each and every limitation of the asserted claims, arranged as in the claims (at least in the alternative), Sun anticipates.

V. OPINIONS REGARDING OBVIOUSNESS

A. Legal Standards

58) My understanding of the law regarding obviousness has been informed by counsel for Lallemand. It is my understanding that a patent claim may be considered obvious, and thus invalid, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which the subject matter pertains.

59) To apply this standard, I am further informed that one must first determine the scope and content of the prior art. This includes all information, including general knowledge, that existed in the same field of art as the subject claims at the time of the alleged invention. One can also consider information which existed in related fields of art to which a POSITA could reasonably have been expected to turn in order to meet the need addressed by the alleged invention of the subject claims. To perform this task, I also must determine the level of skill that a POSITA would have had at the time.

60) After a determination of the scope and content of the prior art and the ordinary level of skill, the next task is to determine the differences between the prior art and the asserted claims. After having done so, the next step is to compare the prior art to the

invention "as a whole." That is, I am instructed to determine whether the invention (as defined by the asserted claims) as a whole is obvious in light of the prior art.

61) Lastly, I am informed that obviousness, like anticipation, must be proven by "clear and convincing" evidence.

B. The Scope and Content of the Prior Art

62) The relevant field of art of the '998 patent is industrial biotechnology. More specifically, the field of art is the industrial production of ethanol from lignocellulosic biomass, so-called "second generation" biofuel production. See, e.g., '998 patent, Col. 1, lns. 34-60. First generation biofuels are based, at least in part, on the fermentation of food crops such as corn, wheat, and sugar beets. While first generation ethanol production is a proven technology, its use and competition for consumption of food crops limits its economic viability as the cost of such fermentation exceeds the value of the ethanol produced. Accordingly, those of skill in this field at the time were interested in obtaining industrial ethanol biofuel production from lignocellulosic matter, such as plant residue like corn stalks which otherwise have marginal economic value.

63) As acknowledged by the inventors of the '998 patent, molecular biology research techniques, including homologous recombination strategies for gene deletion and site-directed mutagenesis, had been in existence well prior to the patent's priority date. *See, e.g.*, '998 patent at Col. 5, lns. 28-29 (citing Sambrook, et al., "Molecular Cloning—A Laboratory Manual," 2d. ed., Vol. 1-3, published in 1989). I thus agree with the inventors that, at the time of their invention, "a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art." See

‘998 patent at Col. 5, lns. 57-60. At least as of that time, a POSITA would have been well familiar with all of the techniques mentioned and disclosed in the ‘998 patent specification, and would have enjoyed a reasonable likelihood of success in creating stable mutations in a great variety of genetic loci in yeast strains, as well as introducing a great variety of heterologous genes into yeast strains.

64) Moreover, the power of molecular genetic techniques like those detailed in manuals such as Sambrook along with the relative ease and low cost with which microorganisms could be cultivated allowed for numerous parallel studies to be conducted in which various combinations of mutagenesis and heterologous gene recombination could be considered and compared.

65) An important facet of yeast bioengineering is that cells can be manipulated to alter metabolic flux of carbon through diverse pathways such that one can drive carbon flux toward ethanol production, longer chain alcohols or fatty acid biosynthesis, among various pathways. Yeast bioengineering can be accomplished by manipulating many, but not all genes. Genes essential for yeast viability are difficult to manipulate, but non-essential genes such as many metabolic pathway genes can be altered in their expression patterns. It is well known in yeast physiology that yeast exhibit a plasticity in that alterations in one metabolic pathway induce changes in metabolic flux toward other pathways. Yeast have a myriad of bypass pathways. See, for example, “The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression,” Strathern, J.N., et al., Cold Spring Harbor Laboratory, 1982. Thus, in ethanol producing yeast cultured under anaerobic conditions, a critical issue for the cells is maintaining redox balance in the NADH/NAD^+ ratio. Glycerol synthesis is one common mechanism to

achieve redox balance, but yeast have other pathways capable of regenerating NAD^+. Research has shown that yeast use the pathway that is energetically most favorable. Thus, metabolic rewiring can occur depending on the carbon source available or heterologous enzymes expression in yeast creating new metabolic options.

66) Below I survey several references that I believe reflect the scope and content of the prior art as of the filing date of the '998 patent. I summarize the teachings of these references below for the purpose of brevity, I incorporate them by reference in their entirety, and reserve the right to opine about them at trial without restriction. These references are identified in a chart in Defendants' First Set of Invalidity Contentions, pages 8-10, and I generally use the shorthand identifiers in that chart. It is my understanding that all of these references have been produced to the plaintiffs.

67) De Virgilio describes a study of the *S. cerevisiae* acetyl-coenzyme A synthetase gene (EC 6.2.1.1). Specifically, De Virgilio discloses that *S. cerevisiae* can use acetate as a carbon source under certain conditions. Under these conditions, free acetate is converted to acetyl-Co A by the ATP-activated catalysis of acetyl-CoA synthetase according to the following equation:

$$\text{Acetate} + \text{CoA} + \text{ATP} \rightarrow \text{Acetyl-CoA} + \text{AMP} + \text{PP}_i$$

De Virgilio also discloses that acetyl-CoA synthetase also is used in ethanol utilization converting the acetate formed by oxidation of ethanol to acetyl-CoA.

68) Björkqvist discloses that Gpd1 and Gpd2 enzymes are both implicated in glycerol formation by *S. cerevisiae* under anaerobic conditions, and that *gpd1Δ/gpd2Δ* double

mutants are unable to grow anaerobically. But addition of acetoin to the growth medium rescued this functionality by regenerating NAD^+ through the formation of butanediol. These researchers concluded that generation of glycerol during fermentation by *S. cerevisiae* is not required *per se*, but rather that some other "redox sink" is acceptable to regenerate NAD^+. Notably, the authors also noted that the "*gpd2*Δ mutant is able to overcome the redox problems [caused by deletion of that gene] by increasing Gpd1 activity, probably by synthesizing more Gpd1 enzyme." An additional way redox homeostasis may be achieved is by reducing biomass production.

69) Ansell discloses that *GPD2* expression is stimulated by anoxic conditions, and that mutants deleted for both *GPD1* and *GPD2* do not produce any detectable glycerol, and also accumulate NADH. However, these cells fail to propagate under anoxic conditions. This phenotype was rescued by addition of acetaldehyde, indicating that that molecule served to regenerate NAD^+ in the absence of glycerol formation through the action of alcohol dehydrogenase to generate ethanol. Notably, the authors also noted that "the enzymes encoded by Gpd1 and Gpd2 appear to be able to substitute for each other, depending on the growth conditions." They also noted that, "when expressed at a high enough level, either isoform is capable of substituting for the other." Yeast lacking Gpd1 have no phenotype under anaerobic conditions, whereas cells lacking Gpd2 are growth impaired and glycerol production is reduced. Thus, Gpd2 is the more important enzyme during anaerobic growth. Importantly, Ansell also noted that cells lacking Gpd2 have increased expression of Gpd1.

70) Taherzadeh discloses that the addition of acetate to *S. cerevisiae* growth media may facilitate formation of acetyl-CoA, reduce formation of NADH, decrease glucose

yield, and increase ethanol yield. As the authors concluded, "it can be said that when treated in a correct way, acetic acid will indeed behave as a friend in the anaerobic conversion of glucose to ethanol by *S. cerevisiae*, rendering both a higher ethanol yield and lower by-product yields." Although acetate increased ethanol yield and diminished glycerol production, biomass was also decreased, most likely due to the consumption of ATP in generating acetyl-CoA.

71) Valadi '98 discloses experimental measurement of anaerobic performance of *gpd1*Δ and *gpd2*Δ mutants of *S. cerevisiae* using a high performance bioreactor. The authors determined that *gpd2*Δ mutants are able to maintain redox balance under anaerobic conditions even with the substantial reduction of glycerol formation, and suggest a possible solution to the NADH accumulation problem. Cells lacking Gpd1 did not exhibit an attenuation in glycerol production. Notably, the authors also discovered that *gpd2*Δ mutants express a higher level of Gpd1 compared to the wild type. *Id*. The authors also discovered that the *gpd2*Δ mutants gave higher final ethanol yields and also had lower final acetate concentration than the wild type.

72) Nissen discloses an attempt to introduce a cytoplasmic transhydrogenase from *Azotobacter vinelandii* (*cth*) into a *gpd1*Δ/*gpd2*Δ double mutant strain of *S. cerevisiae* to rescue NADH reoxidation functionality. The deletion of *GPD1* markedly attenuated GPD activity, whereas deletion of *GPD2* did not result in a decrease in GPD activity under anaerobic conditions. The *gpd2*Δ mutant also showed a diminution in glycerol production unlike the deletion of *gpd1*Δ cells. Cells depleted of Gpd2 were compromised in cell growth. The authors also noted that the *gpd2*Δ mutant exhibited increased *GPD1* expression, and concluded that Gpd1 can partially substitute the physiological role of

Gpd2 during anaerobic growth but this did not lead to restoration of the growth rate. The expression of the transhydrogenase that theoretically could reoxidize NADH in the double mutant failed to restore growth in their strain.

73) Bakker reviewed the then-current (i.e., ca. 2001) state of knowledge of mechanisms for reoxidation of NADH in *S. cerevisiae*, identifying at least five different mechanisms, including alcoholic fermentation and glycerol formation. The authors note that *gpd1*Δ/*gpd2*Δ double mutant strains can grow in the presence of an NADH-oxidizing agent such as acetaldehyde or acetoin.

74) Påhlman reports on the physiological roles of *GPP1* and *GPP2* in *S. cerevisiae*, including the reduction in glycerol formation in *gpp1*Δ/*gpp2*Δ double mutants. The authors note that the addition of acetaldehyde restores anaerobic growth in such mutants and reduces glycerol-3-phosphate pools, indicating that the anaerobic accumulation of NADH causes glycerol-3-phosphate to reach growth-inhibiting levels. This was confirmed in studies in which the addition of acetoin prevented NADH accumulation, but failed to reduce glycerol-3-phosphate levels or rescue anaerobic growth. The authors concluded that accumulation of glycerol-3-phosphate rather than NADH was responsible for the anaerobic growth inhibition of the double mutant.

75) Sonderegger II discloses a metabolically engineered transgenic *S. cerevisiae* strain that catabolizes xylose, a product of hemicellulose-rich plant material, with increased ethanol yield, decreased glycerol yield, and decreased acetate formation. Specifically, the strain includes a transgenic acetaldehyde dehydrogenase and

phosphotransacetylase along with a deletion in the gene for aldehyde dehydrogenase (to reduce acetate accumulation).

76) Zhang ’06 discloses a *fps1*Δ mutant *S. cerevisiae* strain that has increased ethanol yield (10%) and decreased glycerol (19%) and acetate yield (5%) without changes in glucose consumption.

77) Zhang ’08 discloses a *fps1*Δ /*gpd2*Δ, double mutant *S. cerevisiae* strain modified to overexpress Glt1 and Gln1 that increased ethanol yield and decreased yields of both glycerol (42%) and acetic acid (68%). The authors conclude that the redox homeostasis was achieved in part by reduced production of acetic acid.

78) Nevoigt ’08 reports on the (then) progress of metabolic engineering of *S. cerevisiae*. Numerous mutant and transgenic strains capable of unique metabolic end products are discussed. One such class of yeast strains have been modified to better tolerate inhibitory substances in lignocellulose hydrolysates, which products include carboxylic acids (e.g., acetate) and phenols.

79) Guo discloses polyploid “industrial” strains of *S. cerevisiae* in which *GPD1* and *GPD2* were deleted, respectively. The *gpd2*Δ mutant strain exhibited higher ethanol yield and lower glycerol yield (8%), while also maintaining redox balance “in some way.” Acetate concentrations were also much lower (49%) in the *gpd2*Δ mutant strain. Guo therefore speculated “that a decrease in acetate formation is an example of a metabolic adjustment by the cells to minimize the NADH surplus when the glycerol formation capacity is hampered.” Levels of Gpd1 activity were not measured in the *gpd2*Δ mutant strain, however. Redox homeostasis may also occur by reducing biomass synthesis and

that was true with the *gpd2*Δ mutant. These studies were done with an industrial yeast strain unlike the laboratory strains used in earlier reports by Ansell, Bjorkqvist, and Nissen. They reported in data not shown that the transformation of *gpd2*Δ cells with an acid protease from *Neurospora* led to a further diminution in glycerol, likely due to the digestion of proteins into amino acids thereby bypassing the need for amino acid synthesis that generates NADH.

80) Minard discloses *S. cerevisiae* strains that exhibit reduced total [NAD^+/NADH], but an increased ratio of NAD^+ to NADH when grown on acetate. Cells depleted of Idh1 and Idh2 that blocks the NADH generating citric acid isocitrate dehydrogenase resulted in enhanced levels of oxidized NAD^+ when cultured on glucose.

81) Waks discloses a *fdh1Δ*/*fdh2Δ* mutant strain of *S. cerevisiae* in which the genes *pflA*, *pflB*, and *adhE* from *E. coli* were inserted. This strain showed high formate production, which the authors sought to demonstrate could be used to induce increased hydrogen production in *E. coli* in a subsequent step. The authors noted that PFL (pyruvate formate lyase) and AdhE (acetaldehyde dehydrogenase) essentially serve as an alternative fermentation route to the *S. cerevisiae* pyruvate-to-ethanol pathway, which is catalyzed by pyruvate decarboxylases and alcohol dehydrogenases, and also helps maintain redox balance anaerobically by regenerating NAD^+. PFL facilitates acetyl-CoA formation and AdhE converts that to ethanol and reoxidizes NADH. The authors also noted that the addition of AdhE enhanced growth rate and biomass yield, which they speculated may be in part due to the alleviation of anaerobic redox stress by AdhE by increasing the NAD^+/NADH ratio.

82) Bellissimi discloses an engineered xylose-fermenting *S. cerevisiae* strain (RWB 218) based on the *Piromyces* XylA gene and in which the enzymes of the nonoxidative pentose-phosphate pathway have been overexpressed. The authors note the ethanol fuel industry's interest in using lignocellulosic biomass to generate ethanol may be cost effective, but also acknowledge the deleterious effects of high acetate concentrations in lignocellulosic hydrolysates. They therefore set out to investigate the impact of acetic acid on the kinetics and stoichiometry of glucose and xylose on strain RWB 218. The authors noted the stimulatory effect on fermentative yeast performance at low concentrations of acetic acid.

83) Wahlbom discloses a transgenic *S. cerevisiae* strain including genes for overexpressing xylose reductase, xylitol dehydrogenase and xylulokinase and heterologous genes encoding phosphoacetyl transferase and acetaldehyde dehydrogenase. The inventors expressed their intention for the strain to be useful for generation of ethanol from the fermentation of lignocellulosic matter, which includes pentoses such as xylose. The inventors faced the specific problem of regenerating NAD^+ in xylose fermenting strain while also limiting production of glycerol or xylitol. One such known solution was the addition of an acylating acetaldehyde dehydrogenase to the strain. The inventors also foresaw the utility of acetaldehyde dehydrogenase to convert extracellular acetate (a known inhibitor of yeast fermentation) into ethanol.

84) Winkler discloses transgenic yeast strains that are able to metabolize xylose, useful for fermentation of lignocellulosic matter, among other uses.

85) Siddavattam discloses an excisable gene expression cassette for heterologous expression of two different dehydrogenases, including an alcohol dehydrogenase, to rebalance [NAD^+/NADH] in yeast.

86) Nevoigt ‘270 discloses yeast strains having reduced Gpd1 and/or Gpd2 activity with concomitant increased ethanol production and decreased glycerol production. I discuss Nevoigt ‘270 further below.

87) Mueller discloses means for identifying transgenic yeast strains useful for forming butanol (among other products) via acetyl-CoA as an intermediate using (among other things) a heterologous acetylating acetaldehyde dehydrogenase which catalyzes the following reversible reaction:

$$\text{acetaldehyde (AA)} + NAD^+ + \text{CoA} \leftrightarrow \text{acetyl-CoA} + \text{NADH} + H^+$$

88) Campbell discloses yeast strains metabolically engineered for the production of high-value chemicals from free fatty acids. Among the mutations introduced by Campbell is the introduction of the bifunctional AdhE gene from *E. coli* which catalyzes the following reversible reactions:

$$\text{acetyl-CoA} + \text{NADH} \leftrightarrow \text{acetaldehyde} + \text{CoA} + NAD^+$$

$$\text{acetaldehyde} + \text{NADH} \leftrightarrow \text{ethanol} + NAD^+$$

89) Sun discloses transgenic yeasts useful for the industrial production of primary alcohols. Sun also discloses, among other things, introduction of exogenous AdhE from *E. coli* for the reduction of acetyl-CoA to ethanol.

90) Festel discloses transgenic yeast cells useful for production of numerous organic molecules via increased production of acetyl-CoA precursor. One method Festel discloses is via an acetylating acetaldehyde dehydrogenase.

C. The Level of Ordinary Skill in the Art

91) Given the subject matter of the '998 patent, in my opinion, a POSITA would have the equivalent of a Ph.D. in microbiology or biochemistry with relevant knowledge of eukaryotic physiological pathways, preferably those of yeasts capable of both oxidative and fermentative catabolism. Such a person would also have experience or at least familiarity with basic molecular biological techniques such as recombinant DNA technology, gene editing, cloning, and bioengineering.

D. The Differences Between the Prior Art and the Claims as a Whole

92) It is my understanding that DSM is asserting claims 1, 5, and 11 from the '998 patent. I reproduce those claims here, labeling each limitation individually for purposes of comparison with the prior art. In my analysis, I will use the labels to refer to the individual limitations:

> **Claim 1:**
>
> **[a]** Transgenic yeast cells comprising one or more recombinant heterologous, nucleic acid sequences encoding a protein with NAD^{+}-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10),

[b] wherein said cells lack enzymatic activity needed for the NADH-dependent glycerol synthesis, or said cells have a reduced enzymatic activity with respect to the NADH-dependent glycerol synthesis compared to a corresponding wild-type yeast cell, and

[c] wherein said cells are free of NAD-dependent glycerol 3-phosphate dehydrogenase activity or have reduced NAD-dependent glycerol 3-phosphate dehydrogenase activity compared to corresponding wild-type cells, and/or

[d] wherein the cells are either free of glycerol phosphate phosphatase activity or have reduced glycerol phosphate phosphatase activity compared to corresponding wild-type cells, and

[e] which comprise a genomic mutation in at least one gene selected from the group consisting of GPD1, GPD2, GPP1 and GPP2, and

[f] wherein said cells further comprise one or more nucleic acid sequences encoding an acetyl-Coenzyme A synthetase activity (EC 6.2.1.1) and

[g] one or more nucleic acid sequences encoding NAD^+-dependent alcohol dehydrogenase activity (EC 1.1.1.1).

Claim 5:

The cells of claim 1 which are **[h]** *Saccharomycetaceae*, *Kluyveromyces*, *Pichia*, *Zygosaccharomyces*, or *Brettanomyces*.

Claim 11:

The cells of claim 1, wherein **[i]** at least one said mutation is a complete deletion of said gene in comparison to the corresponding wild-type yeast gene.

1. Nevoigt ‘270 in View of Wahlbom and Further in View of Taherzadeh

93) Nevoigt ‘270 addresses a known problem in the art regarding glycerol formation during fermentation of sugars by yeast. See Col. 1, ln. 12 – Col. 2, .ln. 3. Glycerol is an unwanted by-product of industrial ethanol production, and its production consumes carbon that could otherwise be directed to the formation of ethanol, theoretically increasing its yield by 10%. Id.

94) The role played by glycerol formation during fermentation by *S. cerevisiae* was already well known even prior to Nevoigt ‘270: *S. cerevisiae* shunts carbon to the glycerol pathway under fermentative conditions to rebalance cytosolic NAD^+ and NADH concentrations. See Col. 1, lns. 46-51. See Col. 1, lns. 39-62. Likewise, the entire pathway, including all of the enzymes, reactants, and products were also known. It was also known that elimination of both *GPD1* and *GPD2* blocks glycerol synthesis in fermenting *S. cerevisiae*, but is economically disadvantageous because it leads to negligible (or no) biomass formation due to the inability of such double mutants to regenerate NAD^+. See, e.g., Nissen, pp. 464, 467-68. It was also known prior to Nevoigt ‘270 that elimination of both *GPP1* and *GPP2* causes the same problem. See, e.g., Påhlman, pp. 3557-58. Nevoigt ‘270 confirmed these findings. Col. 3, lns. 4-8; Col. 12, lns. 6-8. The contribution that Nevoigt ‘270 made was to down-regulate glycerol

production by disrupting, rather than blocking, the glycerol pathway. This was accomplished by knocking out *GPD2*, *GPP1*, and *GPP2*, and by reducing the expression of *GPD1* by replacing its native promotor with a heterologous weak promoter. Col. 8, lns. 29-65 (creating strain EN-G46a). This resulted in reduced glycerol synthesis and increased ethanol synthesis, while maintaining approximately wild-type biomass production. Col. 12, lns. 25-54. This was in keeping with Nevoigt ‘270’s hypothesis that yeast subject to industrial fermentation conditions could not thrive without some glycerol production. See Col. 2, lns. 58-61.

95) Thus, Nevoigt ‘270 identifies the identical glycerol problem addressed by the ‘998 patent inventors, and discloses a similar solution. In fact, Nevoigt ‘270 teaches virtually the entirety of the asserted claims.

96) Nevoigt ‘270 literally meets limitations [b], [c], and [e] because it discloses a transgenic yeast strain that has at least reduced enzymatic activity needed for NADH-dependent glycerol synthesis and reduced NAD-dependent glycerol 3-phosphate dehydrogenase (“GPD”) activity compared to wild-type cells, and a genomic mutation in at least one of *GPD1* and *GPD2*. Nevoigt ‘270, Col. 3, lns. 9-44; Col. 3, ln. 49 – Col. 5, ln. 9; Col. 5, ln. 58 – Col. 6, ln. 13; Col. 7, lns. 19-65; Col. 8, lns. 10-65; Col. 9, ln. 63 – Col. 10, ln. 49; Col. 11, ln. 61 – Col. 12, ln. 59; Figs. 2-7.

97) Nevoigt ‘270 also literally meets limitations [b], [d] and [e] because it discloses a transgenic yeast strain that has at least reduced enzymatic activity needed for NADH-dependent glycerol synthesis and that is either free of glycerol phosphate phosphatase (“GPP”) activity or has reduced GPP activity compared to corresponding wild-type cells,

and has a genomic mutation in at least one of *GPP1* and *GPP2*. Nevoigt ‘270, Col. 5, lns. 5-39; Col. 8, lns. 11-14; Col. 8, lns. 50-65; Col. 10, ln. 50 – Col. 12, ln. 59; Figs. 2-7.

98) Nevoigt ‘270 literally meets limitations [f] and [g] because wild-type *S. cerevisiae* cells comprise one or more nucleic acid sequences for both an acetyl-coenzyme A synthetase activity and a NAD^+-dependent alcohol dehydrogenase activity, and the strains disclosed in Nevoigt ‘270 are wild-type with respect to these genes. See Nevoigt ‘270, Col. 8, lns. 10-65.

99) Nevoigt ‘270 literally meets limitation [h] because it discloses *S. cerevisiae* yeast strains having the above genes and mutations. Nevoigt ‘270, Col. 5, lns. 40-49; Col. 8, lns. 10-26.

100) Nevoigt ‘270 literally meets limitation [i] because it discloses yeast strains in which at least *GPD2*, *GPP1* and *GPP2* have been deleted. Nevoigt ‘270, Col. 8, lns. 11-14; Col. 10, ln. 50 – Col. 11, ln. 67.

101) Thus, the only limitation of the asserted claims that Nevoigt ‘270 does not disclose is limitation [a] of claim 1, specifying a heterologous nucleic acid sequence encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10). As I mentioned above, the only purpose for this limitation disclosed in the ‘998 patent is to allow reoxidation of NADH when acetyl-CoA is generated from acetate present in the growth medium. See ‘998 patent, Col. 10, lns. 4-16. However, a POSITA at the time would have known not only about the reaction catalyzed by acetaldehyde dehydrogenase, but also that the addition of acetate to the

growth medium could have positive effects on growth of *S. cerevisiae* by promoting decreased NADH formation. See, e.g., Taherzadeh, p. 2658.

102) Taherzadeh teaches that, under certain conditions, acetate may be beneficial for conversion of glucose to ethanol in *S. cerevisiae*. See Taherzadeh, p. 2658. Taherzadeh also teaches that the addition of acetate to growth media may lead to decreased glycerol yield and increased ethanol yield. See *id.* In fact, Taherzadeh teaches that high acetic acid additions to growth medium leads to acetate consumption. See *id.*

103) Nevoigt '270 discusses conditions in which growth media inherently may include increased acetate concentrations. For example, Nevoigt '270, discusses constructing yeast strains that can ferment lignocellulosic matter in addition to conventional feedstock, and that such material might require chemical, enzymatic, or mechanical treatment prior to fermentation. See Col. 6, lns. 28-31, 39-44. A POSITA at the time would have known that the materials and conditions discussed by Nevoigt '270 could introduce acetate and other fermentation inhibitors into the growth medium. See, e.g., Wahlbom, Col. 1, lns. 27-43.

104) Wahlbom discloses transgenic yeast cells adapted to ferment sugars liberated by hydrolysis of lignocellulosic matter. Col. 1, ln. 7 – Col. 2, ln. 11. Also like Nevoigt '270, Wahlbom recognized that glycerol is an unwanted by-product of such fermentation. Col. 5, ln. 66 – Col. 6, ln. 4; Col. 7, lns. 40-43; Col. 8, lns. 9-15. However, Wahlbom explicitly recognized many of the problems that had to be overcome when fermenting these

materials, including the presence of fermentation inhibitors such as phenolics, furan derivatives, and acids (including acetic acid). Col. 1, lns. 27-43.

105) In addition, Wahlbom focused on engineering a yeast strain that could ferment xylose (a component of hemicellulose) to produce ethanol. Col. 2, ln. 48 – Col. 4, ln. 66. Specifically, Wahlbom introduced genetic modifications to strains of *S. cerevisiae* comprising genes for overexpression of xylose reductase, xylitol dehydrogenase, and xylulokinase. Col. 6, ln. 66 – Col. 7, ln. 4; Col. 11, lns. 20-60. However, Wahlbom's mutations introduced a redox imbalance resulting in NAD^+ exhaustion (and hence NADH accumulation). Col. 5, ln. 1 – Col. 6, ln. 31; Col. 6, ln. 66 – Col. 7, ln. 8. Part of Wahlbom's solution to this problem included the introduction of an acetylating acetaldehyde dehydrogenase, as part of a phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, to convert xylulose-5-phosphate to acetyl phosphate and glyceraldehyde phosphate, then acetyl phosphate to acetyl-CoA, and finally to acetaldehyde, restoring NAD^+. Col. 7, lns. 27-33; Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, Ln. 17; Col. 12, ln. 42 – Col. 13, ln. 3.

106) Wahlbom also teaches additional benefits from introducing acetaldehyde dehydrogenase into *S. cerevisiae* besides regeneration of NAD^+. One such benefit is the conversion of acetate to ethanol, leading to increased ethanol yield. Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, ln. 37; Col. 12, ln. 42 – Col. 13, ln. 3. As I noted above, this is similar to the result the inventors of the '998 patent attempted to achieve, and uses an identical enzyme, acetaldehyde dehydrogenase, in the same way to achieve the purpose.

107) Given the above, it is my opinion that the asserted claims are obvious over Nevoigt '270 in view of Wahlbom, and further in view of Taherzadeh. Nevoigt '270 discloses every limitation of the asserted claims except the requirement for a NAD^+-dependent acetylating acetaldehyde dehydrogenase. Wahlbom discloses this missing limitation.

108) Motivation to combine is provided by both Nevoigt '270 and Wahlbom. Both references are directed to improving the economic viability of industrial ethanol fermentation by metabolically engineering strains of *S. cerevisiae* to have increased ethanol production. Both references also teach that glycerol is essentially a contaminant in such fermentation, and its reduction could even result in increased ethanol yield. Both references also discuss using lignocellulosic matter to further increase the economic advantages of industrial ethanol production.

109) While Nevoigt '270 teaches decreasing glycerol production by disrupting the fermentative glycerol pathway, it does not teach oxidation of cytosolic NADH. However, Wahlbom teaches rebalancing the NAD^+/NADH ratio through the phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, in the context of a *S. cerevisiae* strain also possessing a xylose fermentation pathway. The advantages of combining the *gpd2*Δ/*gpp1*Δ/*gpp2*Δ mutant of Nevoigt '270 with the NAD^+ restoring pathway of Wahlbom would have been apparent, as Nevoigt '270's method necessarily results in increased NADH and reduced NAD^+ concentrations. Furthermore, because the lignocellulosic hydrolysates envisioned for use by Wahlbom (and apparently by Nevoigt '270 as well) also contain xylose (a component of hemicellulose), the addition of

a xylose fermentation pathway would have provided even more motivation to combine these references as further economic advantages could be realized.

110) Moreover, in light of Taherzadeh and Wahlbom, a POSITA would have appreciated that the addition of acetate would have allowed for reoxidation of even more NADH, and that lignocellulosic hydrolysates contain appreciable amounts of acetate. Wahlbom's acetaldehyde dehydrogenase pathway would have facilitated consumption of this additional acetate in a yeast strain including Nevoigt '270's disrupted glycerol pathway, increasing production of ethanol and reoxidizing NADH.

111) Accordingly, given the high level of skill in the art in this field at the time, it is my opinion that a POSITA would have found it obvious at least to try to construct a transgenic strain of *S. cerevisiae* with Nevoigt '270's disrupted fermentative glycerol pathway and Wahlbom's xylose and acetaldehyde dehydrogenase pathways because doing so would have enjoyed the advantages of both approaches, and would have been optimized for second generation industrial fermentation in which the growth medium contains glucose, xylose, and acetate. For example, a POSITA could have begun with Wahlbom's TMB3001 and/or TMB3001c strains and simply knocked out (at least) *GPD2*, *GPP1*, and *GPP2* according to Nevoigt '270's method. Such a POSITA would have enjoyed a reasonable expectation of success in so combining these references as all of the relevant methodologies were available for use, and strains were already available with which to work. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

2. Nevoigt '270 in View of Mueller and Further in View of Taherzadeh

112) Mueller teaches transgenic *S. cerevisiae* strains useful for the fermentation of various metabolites (namely butanol) that require acetyl-CoA as a precursor. See p. 1, lns. 5-11. To this end, Mueller teaches disruption of the pyruvate dehydrogenase by-pass (blocking formation of acetate from acetaldehyde via acetaldehyde dehydrogenase) and introduction of a heterologous acetylating acetaldehyde dehydrogenase for converting subsequently accumulated acetaldehyde into acetyl-CoA. See p. 3, ln. 25 – p. 6, ln. 4. Optionally, genes for encoding a recombinant pathway for the formation of butanol may be introduced. See p. 6, ln. 10 – p. 7, ln. 3. Mueller's preferred yeast is *S. cerevisiae* (p. 7, lns. 4-5) and his "most" preferred growth media is hydrolyzed lignocellulosic matter. See p. 20, lns. 19-31.

113) In my opinion, a POSITA at the time would have been motivated to combine Nevoigt '270 with Mueller in light of Taherzadeh. Like Wahlbom, Mueller teaches using a lignocellulosic hydrolysate as growth medium for *S. cerevisiae* fermentation. As explained above, a POSITA would have known that such a hydrolysate contains acetate, and that acetate can be used as a carbon source and increase ethanol production at the expense of glycerol formation. See Taherzadeh, p. 2658. Moreover, although Mueller disrupts the pyruvate dehydrogenase by-pass in order to increase acetyl-CoA formation using an acetylating acetaldehyde dehydrogenase to catalyze the reaction:

acetaldehyde + NAD^+ + CoA → acetyl-CoA + NADH + H^+ (see p. 13, lns. 23-27),

a POSITA would have known that introducing the acetylating acetaldehyde dehydrogenase of Mueller into the yeast strains of Nevoigt '270 and growing them in the presence of a lignocellulosic hydrolysate including acetate would push the reaction in the opposite direction and regenerate NAD^+ consumed due to Nevoigt '270's disrupted glycerol pathway. Motivation to introduce the acetylating acetaldehyde dehydrogenase from Mueller into the cells of Nevoigt '270 would have been provided by the need to regenerate NAD^+ from acetate in a lignocellulosic hydrolysate. As I explained above, the wealth of knowledge of *S. cerevisiae* and relevant biotechnological techniques would have made it at least obvious to try to combine these references in light of the economic incentives involved.

3. Nevoigt '270 in View of Sonderegger II and Further in View of Taherzadeh

114) Sonderegger II teaches a strain of *S. cerevisiae* capable of fermenting xylose. See p. 2892. A problem Sonderegger II encountered introducing a xylose pathway was the limited capacity of such yeast for anaerobic reoxidation of NADH. See *id.* The authors addressed this problem by creating an aldehyde dehydrogenase mutant (TMBALD6c) expressing acetaldehyde dehydrogenase and phosphotransacetylase (creating strain TMBALD6c-p5EHADH2/p4PTA) that had increased ethanol formation. The phosphotransacetylase converts xylulose-5-phosphate to acetyl-phosphate and glyceraldehyde-3-phosphate. See p. 2895-96.

115) Given the above, in my opinion, the asserted claims are obvious over Nevoigt '270 in view of Sonderegger II, and further in view of Taherzadeh. As mentioned above, a POSITA at the time would have been motivated to create *S. cerevisiae* strains that

could ferment lignocellulosic matter. Sonderegger II provides such strains that include a xylose fermentation pathway. See p. 2892. Sonderegger II does not directly address the impact of glycerol formation on the economics of industrial biofuel fermentation, however, but the strains taught by this reference have decreased glycerol formation. As also mentioned above, given the economics of industrial yeast fermentation, a POSITA would have been further motivated to further decrease the glycerol formation rate of Sonderegger II's strains to increase their economic impact, and would have recognized that Sonderegger II's strains already possessed NAD^+ restoring capability. See pp. 1293-96. Such a person would thus have been motivated to introduce the glycerol pathway disrupting genetic mutations from Nevoigt '270 into Sonderegger II's TMBALD6c-p5EHADH2/p4PTA strain to achieve strains that could utilize both glucose and xylose, and also produce more ethanol and less glycerol. Moreover, given the probable presence of acetate in lignocellulosic matter, such a person would have known, via Taherzadeh, that under certain conditions acetate could be beneficial to growth.

116) In my opinion, given the strong economic incentives to create yeast strains able to ferment lignocellulosic matter, it would have been obvious for a POSITA at least to try to construct yeast strains that possessed the beneficial characteristics of both Nevoigt '270 and Sonderegger II. Moreover, given the high level of skill in the art, a POSITA at the time would have enjoyed a reasonable expectation of success because relevant strains had already been created, relevant data were available from both Nevoigt '270 and Sonderegger II, and thus minimal additional experimentation would have been

required to achieve a *S. cerevisiae* strain containing the beneficial characteristics of both references.

4. Zhang in View of Wahlbom and Further in View of Taherzadeh

117) Zhang sought to decrease glycerol formation by *S. cerevisiae* in industrial ethanol fermentation. See p. 620. Specifically, Zhang sought to determine, among other things, the effect of deleting both *FPS1* (encoding a glycerol export channel protein) and *GPD2* on glycerol production. Id., pp. 620-21. Zhang teaches that these genetic modifications modestly improved ethanol yield (+ 9.81%) and decreased glycerol yield (-37.4%). Id., pp.623-25. Zhang also surmised that Gpd1 allowed the cells to continue to synthesize glycerol while maintaining osmotic tolerance. Id., p. 625.

118) Thus, the contribution that Zhang made was to down-regulate glycerol production by disrupting, rather than blocking, the glycerol pathway. This was accomplished by knocking out *FPS1* and *GPD2*. Id., pp. 621-22 (creating strain ZAL69). This resulted in reduced glycerol synthesis and increased ethanol synthesis, while maintaining approximately wild-type biomass production. Id., pp. 623-25.

119) Thus, Zhang identifies an identical problem to one addressed by the ‘998 patent inventors, and discloses a similar solution. Zhang teaches many of the limitations of the asserted claims.

120) Zhang literally meets limitations [b], [c], and [e] because it discloses a yeast strain that has at least reduced enzymatic activity needed for NADH-dependent glycerol synthesis and reduced NAD-dependent glycerol 3-phosphate dehydrogenase (“GPD”)

activity compared to wild-type cells, and a genomic mutation in at least *GPD2*. Zhang, pp. 621-22; Table 1.

121) Zhang literally meets limitations [f] and [g] because wild-type *S. cerevisiae* cells comprise one or more nucleic acid sequences for both an acetyl-coenzyme A synthetase activity and a NAD^+-dependent alcohol dehydrogenase activity, and the strains disclosed in Zhang are wild-type with respect to these genes. See pp. 621-22; Table 1.

122) Zhang literally meets limitation [h] because it discloses *S. cerevisiae* yeast strains having the above genes and mutations. See p. 621.

123) Zhang literally meets limitation [i] because it discloses yeast strains in which at least *GPD2* has been deleted. See pp. 621-22; Table 1.

124) Thus, the only limitations of the asserted claims that Zhang does not disclose are limitation [a], specifying a heterologous nucleic acid sequence encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10), and limitation [d], specifying reduced or no GPP activity. As I mentioned above, the only purpose for limitation [a] disclosed in the '998 patent is to allow reoxidation of NADH when acetyl-CoA is generated from acetate present in the growth medium. See '998 patent, Col. 10, lns. 4-16. However, a POSITA at the time would have known not only about the reaction catalyzed by acetaldehyde dehydrogenase, but also that the addition of acetate to the growth medium could have positive effects on growth of *S. cerevisiae* by promoting decreased NADH formation. See, e.g., Taherzadeh, p. 2658.

125) Taherzadeh teaches that, under certain conditions, acetate may be beneficial for conversion of glucose to ethanol in *S. cerevisiae*. See Taherzadeh, p. 2658. Taherzadeh also teaches that the addition of acetate to growth media may lead to decreased glycerol yield and increased ethanol yield. See *id.* In fact, Taherzadeh teaches that high acetic acid additions to growth medium leads to acetate consumption. See *id.*

126) Wahlbom discloses transgenic yeast cells adapted to ferment sugars liberated by hydrolysis of lignocellulosic matter. Col. 1, ln. 7 – Col. 2, ln. 11. As I mentioned above, in this field of art at the time (and presently, for that matter), there was intense interest in creating yeast strains that can ferment this material. Also like Zhang, Wahlbom recognized that glycerol is an unwanted by-product of fermentation. Col. 5, ln. 66 – Col. 6, ln. 4; Col. 7, lns. 40-43; Col. 8, lns. 9-15. However, Wahlbom explicitly recognized many of the problems that had to be overcome when fermenting these materials, including the presence of fermentation inhibitors such as phenolics, furan derivatives, and acids (including acetic acid). Col. 1, lns. 27-43.

127) In addition, Wahlbom focused on engineering a yeast strain that could ferment xylose (a component of hemicellulose) to produce ethanol. Col. 2, ln. 48 – Col. 4, ln. 66. Specifically, Wahlbom introduced genetic modifications to strains of *S. cerevisiae* comprising genes for overexpression of xylose reductase, xylitol dehydrogenase, and xylulokinase. Col. 6, ln. 66 – Col. 7, ln. 4; Col. 11, lns. 20-60. However, Wahlbom's mutations introduced a redox imbalance resulting in NAD^+ exhaustion (and hence NADH accumulation). Col. 5, ln. 1 – Col. 6, ln. 31; Col. 6, ln. 66 – Col. 7, ln. 8. Part of Wahlbom's solution to this problem included the introduction of an acetylating

acetaldehyde dehydrogenase, as part of a phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, to convert xylulose-5-phosphate to acetyl phosphate and glyceraldehyde-3-phosphate, then acetyl phosphate to acetyl-CoA, and finally to acetaldehyde, restoring NAD^+. Col. 7, lns. 27-33; Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, Ln. 17; Col. 12, ln. 42 – Col. 13, ln. 3.

128) Wahlbom also teaches additional benefits from introducing acetaldehyde dehydrogenase into *S. cerevisiae* besides regeneration of NAD^+. One such benefit is the conversion of acetate to ethanol, leading to increased ethanol yield. Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, ln. 37; Col. 12, ln. 42 – Col. 13, ln. 3. As I noted above, this is similar to the result the inventors of the '998 patent attempted to achieve, and uses an identical enzyme, acetaldehyde dehydrogenase, in the same way to achieve the purpose.

129) Given the above, it is my opinion that the asserted claims are obvious over Zhang in view of Wahlbom and Taherzadeh. Zhang discloses nearly every limitation of the asserted claims except the requirement for a NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (and reduced or no GPP activity, but this limitation is expressed in the alternative to reduced or no GPD activity). Wahlbom discloses this missing limitation.

130) Motivation to combine is provided by both Zhang and Wahlbom. Both references are directed to improving the economic viability of industrial ethanol fermentation by metabolically engineering strains of *S. cerevisiae* to have increased ethanol production. Both references also teach that glycerol is essentially a contaminant in such

fermentation, and its reduction could even result in increased ethanol yield. At least Wahlbom also discusses using lignocellulosic matter to further increase the economic advantages of industrial ethanol production.

131) While Zhang teaches decreasing glycerol production by disrupting the fermentative glycerol pathway, it does not teach oxidation of cytosolic NADH. However, Wahlbom teaches rebalancing the NAD^+/NADH ratio through the phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, in the context of a *S. cerevisiae* strain also possessing a xylose fermentation pathway. The advantages of combining the *gpd2*Δ/*fps1*Δ mutant of Zhang with the NAD^+ restoring pathway of Wahlbom would have been apparent, as Zhang's method could result in increased NADH and reduced NAD^+ concentrations under certain conditions. Furthermore, because the lignocellulosic hydrolysates envisioned for use by Wahlbom also contain xylose (a component of hemicellulose), the addition of a xylose fermentation pathway would have provided even more motivation to combine these references as further economic advantages could be realized.

132) Moreover, in light of Taherzadeh and Wahlbom, a POSITA would have appreciated that the addition of acetate would have allowed for reoxidation of even more NADH, and that lignocellulosic hydrolysates contain appreciable amounts of acetate. Wahlbom's acetaldehyde dehydrogenase pathway would have facilitated consumption of this additional acetate in a yeast strain including Zhang's disrupted glycerol pathway, increasing production of ethanol and reoxidizing NADH.

133) Accordingly, given the high level of skill in the art in this field at the time, it is my opinion that a POSITA would have found it obvious at least to try to construct a transgenic strain of *S. cerevisiae* with Zhang's disrupted fermentative glycerol pathway and at least Wahlbom's acetaldehyde dehydrogenase pathway because doing so would have enjoyed the advantages of both approaches, and would have been optimized for second generation industrial fermentation in which the growth medium contains glucose, xylose, and acetate. For example, a POSITA could have begun with Wahlbom's TMB3001 and/or TMB3001c strains and simply knocked out (at least) *GPD2*, according to Zhang's method. Such a POSITA would have enjoyed a reasonable expectation of success in so combining these references as all of the relevant methodologies were available for use, and strains were already available with which to work. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

5. Zhang in View of Mueller and Further in View of Taherzadeh

134) Mueller teaches transgenic *S. cerevisiae* strains useful for the fermentation of various metabolites (namely butanol) that require acetyl-CoA as a precursor. See p. 1, lns. 5-11. To this end, Mueller teaches disruption of the pyruvate dehydrogenase by-pass (blocking formation of acetate from acetaldehyde via acetaldehyde dehydrogenase) and introduction of a heterologous acetylating acetaldehyde dehydrogenase for converting subsequently accumulated acetaldehyde into acetyl-CoA. See p. 3, ln. 25 – p. 6, ln. 4. Optionally, genes for encoding a recombinant pathway for the formation of butanol may be introduced. See p. 6, ln. 10 – p. 7, ln. 3. Mueller's

preferred yeast is *S. cerevisiae* (p. 7, lns. 4-5) and his "most" preferred growth media is hydrolyzed lignocellulosic matter. See p. 20, lns. 19-31.

135) In my opinion, a POSITA at the time would have been motivated to combine Zhang with Mueller in light of Taherzadeh. Like Wahlbom, Mueller teaches using a lignocellulosic hydrolysate as growth medium for *S. cerevisiae* fermentation. As explained above, a POSITA would have known that such a hydrolysate contains acetate, and that acetate can be used as a carbon source and can enable conversion of glycerol to ethanol. See Taherzadeh, p. 2658. Moreover, although Mueller disrupts the pyruvate dehydrogenase by-pass in order to increase acetyl-CoA formation using an acetylating acetaldehyde dehydrogenase to catalyze the reaction:

acetaldehyde + NAD^+ + CoA → acetyl-CoA + NADH + H^+ (see p. 13, lns. 23-27),

a POSITA would have known that introducing the acetylating acetaldehyde dehydrogenase of Mueller into the yeast strains of Zhang and growing them in the presence of a lignocellulosic hydrolysate would push the reaction in the opposite direction and regenerate NAD^+ consumed due to Zhang's disrupted glycerol pathway. Motivation to introduce the acetylating acetaldehyde dehydrogenase from Mueller into the cells of Zhang would have been provided by the need to regenerate NAD^+ from acetate in a lignocellulosic hydrolysate. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

6. Zhang in View of Sonderegger II and Further in View of Taherzadeh

136) Sonderegger II teaches a strain of *S. cerevisiae* capable of fermenting xylose. See p. 2892. A problem Sonderegger encountered introducing a xylose pathway was the limited capacity of such yeast for anaerobic reoxidation of NADH. See id. The authors addressed this problem by creating an aldehyde dehydrogenase mutant (TMBALD6c) expressing acetaldehyde dehydrogenase and phosphotransacetylase (creating strain TMBALD6c-p5EHADH2/p4PTA) that had increased ethanol formation. See p. 2895-96.

137) Given the above, in my opinion, the asserted claims are obvious over Zhang in view of Sonderegger II and further in view of Taherzadeh. As mentioned above, a POSITA at the time would have been highly motivated to create *S. cerevisiae* strains that could ferment lignocellulosic matter. Sonderegger II provides such strains that include a xylose fermentation pathway. See p. 2892. Sonderegger II does not directly address the impact of glycerol formation on the economics of industrial biofuel fermentation, however, but the strains taught by this reference have decreased glycerol formation. As also mentioned above, given the economics of industrial yeast fermentation, a POSITA would have been further motivated to further decrease the glycerol formation rate of Sonderegger II's strains to increase their economic impact, and would have recognized that Sonderegger II's strains already possessed NAD^+ restoring capability. See pp. 1293-96. Such a person would thus have been motivated to introduce the glycerol pathway disrupting genetic mutations from Zhang into Sonderegger II's TMBALD6c-p5EHADH2/p4PTA strain to achieve strains that could utilize both glucose and xylose, and also produce more ethanol and less glycerol. Moreover, given the probable presence of acetate in lignocellulosic matter, such a

person would have known, via Taherzadeh, that under certain conditions acetate could be beneficial to growth.

138) In my opinion, given the strong economic incentives to create yeast strains able to ferment lignocellulosic matter, it would have been obvious for a POSITA at least to try to construct yeast strains that possessed the beneficial characteristics of both Zhang and Sonderegger II. Moreover, given the high level of skill in the art, a POSITA at the time would have enjoyed a reasonable expectation of success because relevant strains had already been created, relevant data were available from both Zhang and Sonderegger II, and thus minimal additional experimentation would have been required to achieve a *S. cerevisiae* strain containing the beneficial characteristics of both references. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

7. Nissen in View of Wahlbom and Further in View of Taherzadeh

139) Nissen teaches a transgenic *S. cerevisiae* strain in which *GPD1* and *GPD2* are deleted, and containing a gene encoding a cytoplasmic transhydrogenase from *Azotobacter vinelandii*. See pp. 464-65. Because it was already well known that deletion of both *GPD1* and *GPD2* would lead to a severe deficiency in cytosolic NAD^+, the authors hypothesized that the addition of a transhydrogenase could reoxidize NADH, thereby restoring redox balance. See p. 464.

140) Thus, Nissen identifies an identical problem to one addressed by the ‘998 patent inventors, and discloses a similar solution. Nissen also teaches almost all of the limitations of the asserted claims.

141) Nissen discloses limitations [b], [c], and [e] at least because it teaches a transgenic yeast strain lacking, or having reduced, enzymatic activity for NADH-dependent glycerol synthesis because it has at least reduced NAD^+-dependent glycerol-3-phosphate dehydrogenase activity and has a genomic mutation in at least *GPD1* and *GPD2*. See pp. 464-473.

142) Nissen also discloses limitations [f] and [g] because it the disclosed yeast strains are wild-type with respect to at least an acetyl-CoA synthetase activity and an NAD^+-dependent alcohol dehydrogenase activity. See pp. 464-465.

143) Nissen also discloses limitation [h] because the transgenic yeast is *S. cerevisiae*. See pp. 464-465.

144) Nissen also discloses limitation [i] because at least *GPD1* and *GPD2* are deleted.

145) Thus, the only limitations of the asserted claims that Nissen does not disclose are limitation [a], specifying a heterologous nucleic acid sequence encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10), and limitation [d], specifying reduced or no GPP activity. As I mentioned above, the only purpose for limitation [a] disclosed in the ‘998 patent is to allow reoxidation of NADH when acetyl-CoA is generated from acetate present in the growth medium. See ‘998 patent, Col. 10, lns. 4-16. Nissen teaches an attempt to reoxidize NADH using a

different transgenic enzyme. See pp. 464-65. Moreover, a POSITA at the time would have known not only about the reaction catalyzed by acetaldehyde dehydrogenase, but also that the addition of acetate to the growth medium could have positive effects on growth of *S. cerevisiae* by promoting decreased NADH formation. See, e.g., Taherzadeh, p. 2658.

146) Taherzadeh teaches that, under certain conditions, acetate may be beneficial for conversion of glucose to ethanol in *S. cerevisiae*. See Taherzadeh, p. 2658. Taherzadeh also teaches that the addition of acetate to growth media may lead to decreased glycerol yield and increased ethanol yield. See *id.* In fact, Taherzadeh teaches that high acetic acid additions to growth medium leads to acetate consumption. See *id.*

147) Wahlbom discloses transgenic yeast cells adapted to ferment sugars liberated by hydrolysis of lignocellulosic matter. Col. 1, ln. 7 – Col. 2, ln. 11. As I mentioned above, in this field of art at the time, there was intense interest in creating yeast strains that can ferment this material. Also like Nissen, Wahlbom recognized that glycerol is an unwanted by-product of fermentation. Col. 5, ln. 66 – Col. 6, ln. 4; Col. 7, lns. 40-43; Col. 8, lns. 9-15. However, Wahlbom explicitly recognized many of the problems that had to be overcome when fermenting these materials, including the of fermentation inhibitors such as phenolics, furan derivatives, and acids (including acetic acid). Col. 1, lns. 27-43.

148) In addition, Wahlbom focused on engineering a yeast strain that could ferment xylose (a component of hemicellulose) to produce ethanol. Col. 2, ln. 48 – Col. 4, ln. 66.

Specifically, Wahlbom introduced genetic modifications to strains of *S. cerevisiae* comprising genes for overexpression of xylose reductase, xylitol dehydrogenase, and xylulokinase. Col. 6, ln. 66 – Col. 7, ln. 4; Col. 11, lns. 20-60. However, Wahlbom's mutations introduced a redox imbalance resulting in NAD^+ exhaustion (and hence NADH accumulation). Col. 5, ln. 1 – Col. 6, ln. 31; Col. 6, ln. 66 – Col. 7, ln. 8. Part of Wahlbom's solution to this problem included the introduction of an acetylating acetaldehyde dehydrogenase, as part of a phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, to convert xylulose-5-phosphate to acetyl phosphate and glyceraldehyde-3-phosphate, then acetyl phosphate to acetyl-CoA, and finally to acetaldehyde, restoring NAD^+. Col. 7, lns. 27-33; Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, Ln. 17; Col. 12, ln. 42 – Col. 13, ln. 3.

149) Wahlbom also teaches additional benefits from introducing acetaldehyde dehydrogenase into *S. cerevisiae* besides regeneration of NAD^+. One such benefit is the conversion of acetate to ethanol, leading to increased ethanol yield. Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, ln. 37; Col. 12, ln. 42 – Col. 13, ln. 3. As I noted above, this is similar to the result the inventors of the '998 patent attempted to achieve, and uses an identical enzyme, acetaldehyde dehydrogenase, in the same way to achieve the purpose.

150) Given the above, it is my opinion that the asserted claims are obvious over Nissen in view of Wahlbom and Taherzadeh. Nissen discloses nearly every limitation of the asserted claims except the requirement for a NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (and reduced or no GPP activity, but this limitation

is expressed in the alternative to reduced or no GPD activity). Wahlbom discloses this missing limitation.

151) Motivation to combine is provided by both Nissen and Wahlbom. Both references are directed to improving the economic viability of industrial ethanol fermentation by metabolically engineering strains of *S. cerevisiae* to have increased ethanol production. Both references also teach that glycerol is essentially a contaminant in such fermentation, and its reduction could even result in increased ethanol yield. At least Wahlbom also discusses using lignocellulosic matter to further increase the economic advantages of industrial ethanol production.

152) While Nissen teaches decreasing glycerol production by disrupting the fermentative glycerol pathway, it does not teach (successful) oxidation of cytosolic NADH. However, Wahlbom teaches rebalancing the NAD^+/NADH ratio through the phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, in the context of a *S. cerevisiae* strain also possessing a xylose fermentation pathway. The advantages of combining the *gpd1Δ/gpd2*Δ mutant of Nissen with the NAD^+ restoring pathway of Wahlbom would have been apparent, as Nissan's method necessarily results in increased NADH and reduced NAD^+ concentrations. Furthermore, because the lignocellulosic hydrolysates envisioned for use by Wahlbom also contain xylose (a component of hemicellulose), the addition of a xylose fermentation pathway would have provided even more motivation to combine these references as further economic advantages could be realized.

153) Moreover, in light of Taherzadeh and Wahlbom, a POSITA would have appreciated that the addition of acetate would have allowed for reoxidation of even more NADH, and that lignocellulosic hydrolysates contain appreciable amounts of acetate. Wahlbom's acetaldehyde dehydrogenase pathway would have facilitated consumption of this additional acetate in a yeast strain including Nissen's disrupted glycerol pathway, increasing production of ethanol and reoxidation of NADH.

154) Accordingly, given the high level of skill in the art in this field at the time, it is my opinion that a POSITA would have found it obvious at least to try to construct a transgenic strain of *S. cerevisiae* with Nissen's disrupted fermentative glycerol pathway and at least Wahlbom's acetaldehyde dehydrogenase pathway because doing so would have enjoyed the advantages of both approaches, and would have been optimized for second generation industrial fermentation in which the growth medium contains glucose, xylose, and acetate. For example, a POSITA could have begun with Wahlbom's TMB3001 and/or TMB3001c strains and simply knocked out (at least) *GPD1* and *GPD2*, according to Nissen's method. Such a POSITA would have enjoyed a reasonable expectation of success in so combining these references as all of the relevant methodologies were available for use, and strains were already available with which to work. Given the nature of *S. cerevisiae*, including its plasticity and the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

8. Nissen in View of Mueller and Further in View of Taherzadeh

155) Mueller teaches transgenic *S. cerevisiae* strains useful for the fermentation of various metabolites (namely butanol) that require acetyl-CoA as a precursor. See p. 1, lns. 5-11. To this end, Mueller teaches disruption of the pyruvate dehydrogenase by-pass (blocking formation of acetate from acetaldehyde via acetaldehyde dehydrogenase) and introduction of a heterologous acetylating acetaldehyde dehydrogenase for converting subsequently accumulated acetaldehyde into acetyl-CoA. See p. 3, ln. 25 – p. 6, ln. 4. Optionally, genes for encoding a recombinant pathway for the formation of butanol may be introduced. See p. 6, ln. 10 – p. 7, ln. 3. Mueller's preferred yeast is *S. cerevisiae* (p. 7, lns. 4-5) and his "most" preferred growth media is hydrolyzed lignocellulosic matter. See p. 20, lns. 19-31.

156) Taherzadeh teaches that, under certain conditions, acetate may be beneficial for conversion of glucose to ethanol in *S. cerevisiae*. See Taherzadeh, p. 2658. Taherzadeh also teaches that the addition of acetate to growth media may lead to decreased glycerol yield and increased ethanol yield. See *id.* In fact, Taherzadeh teaches that high acetic acid additions to growth medium leads to acetate consumption. See *id.*

157) In my opinion, a POSITA at the time would have been motivated to combine Nissen with Mueller in light of Taherzadeh. Like Wahlbom, Mueller teaches using a lignocellulosic hydrolysate as growth medium for *S. cerevisiae* fermentation. As explained above, a POSITA would have known that such a hydrolysate contains acetate, and that acetate can be used as a carbon source and can enable conversion of glycerol to ethanol. See Taherzadeh, p. 2658. Moreover, although Mueller disrupts the pyruvate dehydrogenase by-pass in order to increase acetyl-CoA formation using an

acetylating acetaldehyde dehydrogenase to catalyze the reaction: acetaldehyde + NAD^+ + CoA → acetyl-CoA + NADH + H^+ (see p. 13, lns. 23-27), a POSITA would have known that introducing the acetylating acetaldehyde dehydrogenase of Mueller into the yeast strains of Nissen and growing them in the presence of a lignocellulosic hydrolysate would push the reaction in the opposite direction and regenerate NAD^+ consumed due to Nissen's disrupted glycerol pathway. Motivation to introduce the acetylating acetaldehyde dehydrogenase from Mueller into the cells of Nissen would have been provided by the need to regenerate NAD^+ from acetate in a lignocellulosic hydrolysate. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

9. Nissen in View of Sonderegger II and Further in View of Taherzadeh

158) Sonderegger II teaches a strain of *S. cerevisiae* capable of fermenting xylose. See p. 2892. A problem Sonderegger encountered introducing a xylose pathway was the limited capacity of such yeast for anaerobic reoxidation of NADH. See id. The authors addressed this problem by creating an aldehyde dehydrogenase mutant (TMBALD6c) expressing acetaldehyde dehydrogenase and phosphotransacetylase (creating strain TMBALD6c-p5EHADH2/p4PTA) that had increased ethanol formation. See p. 2895-96.

159) Given the above, in my opinion, the asserted claims are obvious over Nissen in view of Sonderegger II and further in view of Taherzadeh. As mentioned above, a POSITA at the time would have been highly motivated to create *S. cerevisiae* strains

that could ferment lignocellulosic matter. Sonderegger II provides such strains that include a xylose fermentation pathway. See p. 2892. Sonderegger II does not directly address the impact of glycerol formation on the economics of industrial biofuel fermentation, however, but the strains taught by this reference have decreased glycerol formation. As also mentioned above, given the economics of industrial yeast fermentation, a POSITA would have been further motivated to further decrease the glycerol formation rate of Sonderegger II's strains to increase their economic impact, and would have recognized that Sonderegger II's strains already possessed NAD^+ restoring capability. See pp. 1293-96. Such a person would thus have been motivated to introduce the glycerol pathway disrupting genetic mutations from Nissen into Sonderegger II's TMBALD6c-p5EHADH2/p4PTA strain to achieve strains that could utilize both glucose and xylose, and also produce more ethanol and less glycerol.

160) Taherzadeh teaches that, under certain conditions, acetate may be beneficial for conversion of glucose to ethanol in *S. cerevisiae*. See Taherzadeh, p. 2658. Taherzadeh also teaches that the addition of acetate to growth media may lead to decreased glycerol yield and increased ethanol yield. See *id.* In fact, Taherzadeh teaches that high acetic acid additions to growth medium leads to acetate consumption. See *id.*

161) In my opinion, given the strong economic incentives to create yeast strains able to ferment lignocellulosic matter, it would have been obvious for a POSITA at least to try to construct yeast strains that possessed the beneficial characteristics of both Nissen and Sonderegger II. Moreover, given the high level of skill in the art, a POSITA at the time would have enjoyed a reasonable expectation of success because relevant strains

had already been created, relevant data were available from both Nissen and Sonderegger II, and thus minimal additional experimentation would have been required to achieve a *S. cerevisiae* strain containing the beneficial characteristics of both references. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

10. Guo in View of Wahlbom and Further in View of Taherzadeh

162) Guo is yet another reference that seeks to lower glycerol yield and raise ethanol yield in *S. cerevisiae*, specifically for use in industrial ethanol production. See pp. 287-88. Guo notes that, unlike previous academic work, genetic modification of industrial *S. cerevisiae* strains is more challenging because of polyploidy. See *id.* The authors sought to show, and claim to have shown, that deletion of either *GPD1* or *GPD2* in industrial *S. cerevisiae* strains can improve ethanol yield. See *id.* Analyses of Gou's results demonstrate that polyploid strains ANG1 (*gpd1*Δ) and ANG2 (*gpd2*Δ) did indeed have higher ethanol yield, although glycerol yield was less affected. See, e.g., Table 1. It is also notable that acetate yield was significantly reduced in both strains. See *id.* The authors also report construction of a third strain of ANG2 transformed with the *AP* gene from *Neurospora crassa*, which codes for a proteinase. See p. 291.

163) Thus, Guo identifies the identical glycerol problem to one addressed by the '998 patent inventors, and discloses a similar solution. Guo also teaches most of the limitations of the asserted claims.

164) Guo discloses limitations [b], [c], and [e] at least because it teaches a transgenic yeast strain lacking, or having reduced, enzymatic activity for NADH-dependent glycerol synthesis because it has at least reduced NAD-dependent glycerol-3-phosphate dehydrogenase activity and has a genomic mutation in at least *gpd1* or *gpd2*. See pp. 289-91.

165) Guo also discloses limitations [f] and [g] because the disclosed yeast strains are wild-type with respect to at least an acetyl-CoA synthetase activity and an NAD^+-dependent alcohol dehydrogenase activity. See pp. 288-89.

166) Guo also discloses limitation [h] because the yeast is *S. cerevisiae*. See pp. 288-89.

167) Guo also discloses limitation [i] because at least *GPD1* or *GPD2* is deleted.

168) Thus, the only limitations of the asserted claims that Guo does not disclose are limitation [a], specifying a heterologous nucleic acid sequence encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10), and limitation [d], specifying reduced or no GPP activity. As I mentioned above, the only purpose for limitation [a] disclosed in the '998 patent is to allow reoxidation of NADH when acetyl-CoA is generated from acetate present in the growth medium. See '998 patent, Col. 10, lns. 4-16. Moreover, a POSITA at the time would have known not only about the reaction catalyzed by acetaldehyde dehydrogenase, but also that the addition of acetate to the growth medium could have positive effects on growth of *S. cerevisiae* by promoting decreased NADH formation. See, e.g., Taherzadeh, p. 2658.

169) Taherzadeh teaches that, under certain conditions, acetate may be beneficial for conversion of glucose to ethanol in *S. cerevisiae*. See Taherzadeh, p. 2658. Taherzadeh also teaches that the addition of acetate to growth media may lead to decreased glycerol yield and increased ethanol yield. See *id.* In fact, Taherzadeh teaches that high acetic acid additions to growth medium leads to acetate consumption. See *id.*

170) Wahlbom discloses transgenic yeast cells adapted to ferment sugars liberated by hydrolysis of lignocellulosic matter. Col. 1, ln. 7 – Col. 2, ln. 11. As I mentioned above, in this field of art at the time, there was intense interest in creating yeast strains that can ferment this material. Also like Guo, Wahlbom recognized that glycerol is an unwanted by-product of fermentation. Col. 5, ln. 66 – Col. 6, ln. 4; Col. 7, lns. 40-43; Col. 8, lns. 9-15. However, Wahlbom explicitly recognized many of the problems that had to be overcome when fermenting these materials, including the presence of fermentation inhibitors such as phenolics, furan derivatives, and acids (including acetic acid). Col. 1, lns. 27-43.

171) In addition, Wahlbom focused on engineering a yeast strain that could ferment xylose (a component of hemicellulose) to produce ethanol. Col. 2, ln. 48 – Col. 4, ln. 66. Specifically, Wahlbom introduced genetic modifications to strains of *S. cerevisiae* comprising genes for overexpression of xylose reductase, xylitol dehydrogenase, and xylulokinase. Col. 6, ln. 66 – Col. 7, ln. 4; Col. 11, lns. 20-60. However, Wahlbom's mutations introduced a redox imbalance resulting in NAD^+ exhaustion (and hence NADH accumulation). Col. 5, ln. 1 – Col. 6, ln. 31; Col. 6, ln. 66 – Col. 7, ln. 8. Part of Wahlbom's solution to this problem included the introduction of an acetylating

acetaldehyde dehydrogenase, as part of a phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, to convert xylulose-5-phosphate to acetyl phosphate and glyceraldehyde phosphate, then acetyl phosphate to acetyl-CoA, and finally to acetaldehyde, restoring NAD^+. Col. 7, lns. 27-33; Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, Ln. 17; Col. 12, ln. 42 – Col. 13, ln. 3.

172) Wahlbom also teaches additional benefits from introducing acetaldehyde dehydrogenase into *S. cerevisiae* besides regeneration of NAD^+. One such benefit is the conversion of acetate to ethanol, leading to increased ethanol yield. Col. 8, lns. 17-37; Col. 10, ln. 48 – Col. 11, ln. 37; Col. 12, ln. 42 – Col. 13, ln. 3. As I noted above, this is similar to the result the inventors of the '998 patent attempted to achieve, and uses an identical enzyme, acetaldehyde dehydrogenase, in the same way to achieve the purpose.

173) Given the above, it is my opinion that the asserted claims are obvious over Guo in view of Wahlbom and Taherzadeh. Guo discloses nearly every limitation of the asserted claims except the requirement for a NAD+-dependent acetylating acetaldehyde dehydrogenase activity (and reduced or no GPP activity, but this limitation is expressed in the alternative to reduced or no GPD activity). Wahlbom discloses this missing limitation.

174) Motivation to combine is provided by both Guo and Wahlbom. Both references are directed to improving the economic viability of industrial ethanol fermentation by metabolically engineering strains of *S. cerevisiae* to have increased ethanol production. Both references also teach that glycerol is essentially a contaminant in such

fermentation, and its reduction could even result in increased ethanol yield. At least Wahlbom also discusses using lignocellulosic matter to further increase the economic advantages of industrial ethanol production.

175) While Guo teaches decreasing glycerol production by disrupting the fermentative glycerol pathway, it does not teach oxidation of cytosolic NADH. However, Wahlbom teaches rebalancing the NAD^+/NADH ratio through the phosphoketolase-phosphotransacetylase-acetaldehyde dehydrogenase pathway, in the context of a *S. cerevisiae* strain also possessing a xylose fermentation pathway. The advantages of combining the *gpd1*Δ and *gpd2*Δ mutants of Guo with the NAD^+ restoring pathway of Wahlbom would have been apparent, as Guo's method necessarily results in increased NADH and reduced NAD^+ concentrations. Furthermore, because the lignocellulosic hydrolysates envisioned for use by Wahlbom also contain xylose (a component of hemicellulose), the addition of a xylose fermentation pathway would have provided even more motivation to combine these references as further economic advantages could be realized.

176) Moreover, in light of Taherzadeh and Wahlbom, a POSITA would have appreciated that the addition of acetate would have allowed for reoxidation of even more NADH, and that lignocellulosic hydrolysates contain appreciable amounts of acetate. Wahlbom's acetaldehyde dehydrogenase pathway would have facilitated consumption of this additional acetate in a yeast strain including Guo's disrupted glycerol pathway, increasing production of ethanol and reoxidation of NADH.

177) Accordingly, given the high level of skill in the art in this field at the time, it is my opinion that a POSITA would have found it obvious to try to construct a transgenic strain of *S. cerevisiae* with Guo's disrupted fermentative glycerol pathway and at least Wahlbom's acetaldehyde dehydrogenase pathway because doing so would have enjoyed the advantages of both approaches, and would have been optimized for second generation industrial fermentation in which the growth medium contains glucose, xylose, and acetate. For example, a POSITA could have begun with Wahlbom's TMB3001 and/or TMB3001c strains and simply knocked out (at least) *GPD1* and *GPD2*, according to Guo's method. Such a POSITA would have enjoyed a reasonable expectation of success in so combining these references as all of the relevant methodologies were available for use, and strains were already available with which to work. Given the nature of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

11. Guo in View of Mueller and Further in View of Taherzadeh

178) Mueller teaches transgenic *S. cerevisiae* strains useful for the fermentation of various metabolites (namely butanol) that require acetyl-CoA as a precursor. See p. 1, lns. 5-11. To this end, Mueller teaches disruption of the pyruvate dehydrogenase by-pass (blocking formation of acetate from acetaldehyde via acetaldehyde dehydrogenase) and introduction of a heterologous acetylating acetaldehyde dehydrogenase for converting subsequently accumulated acetaldehyde into acetyl-CoA. See p. 3, ln. 25 – p. 6, ln. 4. Optionally, genes for encoding a recombinant pathway for the formation of butanol may be introduced. See p. 6, ln. 10 – p. 7, ln. 3. Mueller's

preferred yeast is *S. cerevisiae* (p. 7, lns. 4-5) and his "most" preferred growth media is hydrolyzed lignocellulosic matter. See p. 20, lns. 19-31.

179) In my opinion, a POSITA at the time would have been motivated to combine Guo with Mueller in light of Taherzadeh. Like Wahlbom, Mueller teaches using a lignocellulosic hydrolysate as growth medium for *S. cerevisiae* fermentation. As explained above, a POSITA would have known that such a hydrolysate contains acetate, and that acetate can be used as a carbon source and can enable conversion of glycerol to ethanol. See Taherzadeh, p. 2658. Moreover, although Mueller disrupts the pyruvate dehydrogenase by-pass in order to increase acetyl-CoA formation using an acetylating acetaldehyde dehydrogenase to catalyze the reaction: acetaldehyde + NAD^+ + CoA → acetyl-CoA + NADH + H^+ (see p. 13, lns. 23-27), a POSITA would have known that introducing the acetylating acetaldehyde dehydrogenase of Mueller into the yeast strains of Guo and growing them in the presence of a lignocellulosic hydrolysate would push the reaction in the opposite direction and regenerate NAD^+ consumed due to Guo's disrupted glycerol pathway. Motivation to introduce the acetylating acetaldehyde dehydrogenase from Mueller into the cells of Guo would have been provided by the need to regenerate NAD^+ from acetate in a lignocellulosic hydrolysate. Given the plasticity of *S. cerevisiae*, including the ease with which it can be cultivated and genetically modified, undue experimentation would not have been required.

12. Guo in View of Sonderegger II and Further in View of Taherzadeh

180) Sonderegger II teaches a strain of *S. cerevisiae* capable of fermenting xylose. See p. 2892. A problem Sonderegger encountered introducing a xylose pathway was

the limited capacity of such yeast for anaerobic reoxidation of NADH. See id. The authors addressed this problem by creating an aldehyde dehydrogenase mutant (TMBALD6c) expressing acetaldehyde dehydrogenase and phosphotransacetylase (creating strain TMBALD6c-p5EHADH2/p4PTA) that had increased ethanol formation. See p. 2895-96.

181) Given the above, in my opinion, the asserted claims are obvious over Guo in view of Sonderegger II. As mentioned above, a POSITA at the time would have been highly motivated to create *S. cerevisiae* strains that could ferment lignocellulosic matter. Sonderegger II provides such strains that include a xylose fermentation pathway. See p. 2892. Sonderegger II does not directly address the impact of glycerol formation on the economics of industrial biofuel fermentation, however, but the strains taught by this reference have decreased glycerol formation. As also mentioned above, given the economics of industrial yeast fermentation, a POSITA would have been further motivated to further decrease the glycerol formation rate of Sonderegger II's strains to increase their economic impact, and would have recognized that Sonderegger II's strains already possessed NAD^+ restoring capability. See pp. 1293-96. Such a person would thus have been motivated to introduce the glycerol pathway disrupting genetic mutations from Guo into Sonderegger II's TMBALD6c-p5EHADH2/p4PTA strain to achieve strains that could utilize both glucose and xylose, and also produce more ethanol and less glycerol. Moreover, given the likely presence of acetate in lignocellulosic matter, such a person would have known, via Taherzadeh, that under certain conditions acetate could be beneficial to growth.

182) In my opinion, given the strong economic incentives to create yeast strains able to ferment lignocellulosic matter, it would have been obvious for a POSITA at least to try to construct yeast strains that possessed the beneficial characteristics of both Guo and Sonderegger II. Moreover, given the high level of skill in the art, a POSITA at the time would have enjoyed a reasonable expectation of success because relevant strains had already been created, relevant data were available from both Guo and Sonderegger II, and thus minimal additional experimentation would have been required to achieve an industrial *S. cerevisiae* strain containing the beneficial characteristics of both references.

13. All References Without Acetate Requirement

183) As explained above, given the invention disclosed in the '998 patent specification and the patentee's arguments before the Patent Office, it is my opinion that a POSITA would have understood the transgenic yeast cells of the asserted claims to require acetate as a carbon source. However, it is my understanding that DSM contends that the asserted claims are not so limited. If DSM's interpretation is adopted by the Court, it is my opinion that the asserted claims are invalid as indefinite. This opinion is explained below. Moreover, if DSM's interpretation is adopted by the Court, then my opinions above regarding obviousness still stand, given that the claims do not require the absence of acetate. In addition, if DSM's interpretation is adopted by the Court, it is also my opinion the Taherzadeh reference would become unnecessary for any of the above combinations, and that the motivations to combine would be provided solely by the primary and secondary references identified above, for the reasons given above (minus reference to Taherzadeh and acetate, of course). Moreover, if the yeast cells of claim 1

do not require acetate to grow, it is my opinion that the patentees' arguments to the Patent Office were incorrect, the claims should not have been issued over the combination of Valadi and Sonderegger, and they are invalid as obvious over that combination, as explained by the Examiner. See above.

E. Secondary Considerations of Nonobviousness

184) It is my understanding that part of the obviousness analysis entails so-called secondary considerations of nonobviousness. These include such factors as commercial success, long felt but unmet need, and praise in the industry. I am also informed that, to constitute evidence of nonobviousness, there must be a "nexus," or discernible connection, between such factors and the claimed invention. It is also my understanding that, in the usual process the patent applicant may raise secondary considerations of nonobviousness in response to a "*prima facia*" case of obviousness raised by the patent examiner.

185) At this point, because I am not aware of what, if any, evidence of secondary considerations of nonobviousness the plaintiff intends to introduce, I offer no opinion regarding these considerations. However, I reserve the right to opine regarding any evidence or opinions that the plaintiff or its experts offers regarding secondary considerations of nonobviousness.

VI. OPINIONS REGARDING INDEFINITENESS

A. Legal Standards

186) My understanding of the law regarding indefiniteness has been informed by counsel for Lallemand. I am informed that patent claims must both be enabled and have

definite meaning to a POSITA. In addition, the patent specification must contain a written description of the invention such that a POSITA would understand that the inventor was actually in possession of what was claimed. To satisfy the enablement requirement, a patent specification must contain a written description of the invention, including the manner and process of making and using it, that is complete and clear enough to enable a POSITA to make and use the invention without undue experimentation. I am further informed that, to be definite, patent claims must particularly point out and distinctly claim the subject matter, which the inventor regards as his invention. A patent claim is indefinite if, read in light of the specification and the prosecution history, it fails to inform, with reasonable certainty, a POSITA at the time of invention about the scope of the invention.

187) Whether a claim is definite depends on how the words and phrases in the claim are interpreted. I have been informed that I must use the Court's claim construction ruling in my analysis. Accordingly, I do not provide an opinion regarding the correct constructions of the claim terms, only whether the words of the claims and the Court's constructions reasonably convey the meaning and scope of the claims to a POSITA.

B. Analysis

188) Claim 1 specifies (among other things) "wherein said cells lack enzymatic activity needed for the NADH-dependent glycerol synthesis, or said cells have a reduced enzymatic activity with respect to the NADH-dependent glycerol synthesis." It is my understanding that DSM contends that the term "enzymatic activity" would be construed by a POSITA to mean "the amount of enzyme that will convert a specific amount of substrate to product in a specific amount of time." (DSM's Opening Claim Construction

Brief Nonbinding Mediation December 8 2016, p. 6). While DSM's definition is broadly true, I disagree that a POSITA would consider it sufficient. In fact, as stated, DSM's definition is so broad as to be essentially meaningless.

189) The phrase "enzymatic activity" refers to the ability of an enzyme to catalyze a chemical reaction. The activity is that it lowers the energy barrier between substrate and product. The enzyme activity within a cell is dictated by the amount of the enzyme, whether allosteric activators or inhibitors are present in the cytoplasm, and whether the enzyme has any post-translational modifications that modulate activity, and the specific conditions (e.g., temperature, intracellular pH). The specific activity of an enzyme refers to the catalytic activity present in a given quantity of that specific protein. Thus, meaningful measurements of enzymatic activity require more information than simply "the amount of enzyme that will convert a specific amount of substrate to product in a given time."

190) The prior art itself demonstrates the importance of specifying reaction conditions to the determination of enzymatic activity. For example, Verduyn sought to determine (among other things) the effect of weak acids on the respiratory enzymatic activity of yeasts. See p. 502. Verduyn performed a number of different assays to determine enzymatic activity of a number of different enzymes, including spectrophotometric assays and other assays as taught by Postma, et al. (1989a). See pp. 502-503. As a POSITA would appreciate, all of these assays specify the conditions under which they are performed. See id. Otherwise, results could not be replicated.

191) Other prior art references are in accord, and provide further evidence that those of skill in the art expect measurements of enzymatic activity to occur under specific

conditions. See, e.g., van den Berg ’95, p. 28954; Påhlman, p. 3560; Cronwright, p. 4450.

192) The ‘998 patent specification itself (which I understand is also directed to those skilled in the art) appears also to confirm the expectation of a POSITA to be given specific conditions under which to determine enzymatic activity. For example, when defining “functional homologue,” the inventors refer those skilled in the art to a specific *in silico* similarity analyses. See ‘998 patent, Col. 6, ln. 47 – Col. 7, ln. 6. Moreover, in the examples through which the inventors sought to show those of skill in the art how to achieve their invention, “Enzyme Activity Assays” are specified, including specific preparation procedures (by reference to Abbott, et al. and Blomberg and Adler (1989)), temperature, and pH. See Col. 20, lns. 20-44.

193) It is my understanding that scientists at Lallemand performed the same glycerol-3-phosphate dehydrogenase assay on the accused products as that set forth by the cited Blomberg and Adler reference (to the extent the necessary parameters could be determined), and achieved results showing an increased, rather than deceased, enzymatic activity. It is noteworthy that Lallemand performed the assay using whole yeast extract, expressing the activity normalized to total cellular protein. It is also my understanding that these results were shared with representatives of DSM. However, I am also informed that DSM dismissed these results as being irrelevant to the claimed invention. This is confusing, because if a particular yeast extract containing glycerol-3-phosphate dehydrogenase can be subjected to the very test set forth in the ‘998 patent, but the results are meaningless to the determination of whether that yeast strain falls

within the scope of the asserted claims, then the definition of "enzymatic activity" would appear to be at the sole discretion of the patentee.

194) For example, DSM's infringement position appears to be that any correlation between the deletion of *GPD2* in *S. cerevisiae* and decreased glycerol synthesis necessarily implies reduced (or no) enzymatic activity needed for the NADH-dependent glycerol synthesis. However, it is a well-known principle that correlation does not establish cause-and-effect. In my opinion, the reduced glycerol synthesis exhibited by the accused products is due to shunting carbon to the more energetically favorable pyruvate-formate lyase pathway, and not to reduced NADH-dependent glycerol synthesis activity, and the Blomberg assay as performed by Lallemand bears this out.

195) Given the above, it is my opinion that, if DSM's understanding of the scope of the asserted claims is correct, a POSITA would be unable to determine the proper scope of the asserted claims with reasonable certainty, given that the only test for glycerol-3-phosphate enzyme activity set forth by the patentees would be irrelevant, and no other assay methods are disclosed. A POSITA would therefore be left to select one of the myriad existing assay methods essentially at his or her own discretion, resulting in inconsistent and potentially contradictory results. Such a POSITA would therefore not be able to determine with reasonable certainty if a particular yeast strain had reduced glycerol-3-phosphate phosphatase activity so as to fall within the scope of the asserted claims. Given my understanding of the law, it is therefore my opinion that the claims are thus rendered indefinite.

196) In addition, as I explained above with respect to my review of the '998 patent and prosecution history, the yeast of the claimed invention requires the presence of

acetate in its growth medium. As I explained above, this requirement is specified throughout the ‘998 patent specification, and the yeast developed in the only detailed example in the patent was unable to grow except when the growth medium was supplemented with 2.0 g/L acetic acid. See Col. 21, lns. 23-31. Moreover, while attempting to advance the ‘998 patent application to issuance, the inventors clearly informed the examiner that the yeast of their invention required acetate. See above. In my opinion, this is undeniable not only in light of the clear and unmistakable statements made, but also in the examiner’s reliance on them. See above.

197) It is my understanding that DSM now takes the position that yeast strains according to the asserted claims do not require acetate in order to grow. If this is the case, it is my opinion that the asserted claims are not supported by the specification. The transgenic yeast of the asserted claims clearly require inclusion of one or more heterologous nucleic acid sequences encoding a protein with NAD^+-dependent acetylating acetaldehyde dehydrogenase activity (EC 1.2.1.10), but the asserted claims themselves do not specify the purpose for this gene. Thus, a POSITA would look to the specification to determine its purpose. As explained above, the only purpose disclosed in the specification is to allow reoxidation of NADH when acetyl-CoA is generated from acetate present in the growth medium. See Col. 10, lns. 13-16. As mentioned above, a review of the prosecution history clearly supports this assessment.

198) Accordingly, it is my opinion that, should the asserted claims be construed so as not to require the claimed transgenic yeast to require acetate for growth, then they are indefinite because the invention so claimed is not described in the specification such

that a POSITA would reasonably understand that the inventors had actually invented what is claimed.

VII. CONCLUSION

437. The foregoing opinions and bases for them are current to the date of my signature below, and are based on the information disclosed in this case up to this date. I reserve the right to supplement my opinions in light of further information disclosed in the case. I also reserve the right to supplement my report to rebut any opinions expressed by any experts retained by the plaintiff.

DATE: May 26, 2017

Dennis Winge

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Dennis Winge